KATHOLIEKE UNIVERSITEIT TE LEUVEN

FACULTY OF MEDICINE
DEPARTMENT OF HUMAN BIOLOGY
DIVISION OF MICROBIOLOGY
REGA INSTITUTE
Dir. : PROF. P. DE SOMER

PURIFICATION AND CHARACTERIZATION OF MOUSE INTERFERON

by

IRWIN A. BRAUDE

Thesis submitted in fulfillment
of the requirements for the degree of
« Doctor in de Wetenschappen »

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Dedication :

To Hope —

All Beginnings are Hard.

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GENERAL INTRODUCTION

Since its serendipitous discovery, the interferon system has not only been of interest to animal virologists, but also to molecular biologists, cell biologists, immunologists, and oncologists. Indeed, interferon may prove more than just an antiviral agent (although this still remains the most effective function), but rather as a complex host-regulatory-defense mechanism.

Interferon, while induced in the presence of most viruses, may also be induced by a variety of substances of non-viral origin, including synthetic compounds. It is defined by function, where one unit represents that amount which can reduce the effects of the virus by 50 %. Its description is vague : a protein, which is stable at pH 2.0, is found in the body- or tissue culture as a result of some stimulating substance, is usually (there are exceptions) most active on homologous cells, and is active against a broad spectrum of both DNA and RNA viruses. The antiviral activity of interferon cannot result from a non-specific cytotoxic effect, but should be mediated by some specific intracellular event, the exact nature of which is still not known. This definition may prove to encompass more than just one type of molecule, but rather a whole class of molecules.

The establishment of hybrid cell-lines has led to a genetic description of both the production and mechanism of action of interferon. Its messenger-RNA has been isolated from a variety of cell species, and its study has not only served as a means of understanding the production of interferon, but has proven a useful probe in describing various cellular regulatory functions. The isolation of its messenger may also lead to the eventual "cloning" of interferon.

A description of the regulatory molecules involved in the induction of the antiviral state has also become clearer. A protein kinase(s), a nuclease(s), and a low molecular weight oligonucleotide seem likely to serve some role. Many useful models, both in vivo and in vitro have been developed, whereby the effects on both cellular and viral processes can be examined.

Interferon itself, has been successfully separated from many of its neighbours. A description of its size and partial chemical characterization

has been made, and with the advent of obtaining pure preparations, both a structural and functional description of the molecule may soon be possible.

Interferon-containing preparations have also been reported to have such non-antiviral activities, as effects on : interferon synthesis (priming and blocking); enhancement of the cytotoxic effects of double-stranded RNAs; cell surfaces, cell growth, and the expression of other cellular functions; and, cells associated with the immune system.

Despite the advances and insights which have been reached in the discipline, the two basic questions, what is interferon, and what is its relationship to its host still remain unanswered. The purpose of this thesis work was to gain a better understanding of the structure-function relationship of the interferon system. This was examined by three different approaches : (i), by comparing the antigenic and functional similarities between mouse and human interferons, employing anti-interferon antibody as an immunochemical probe; (ii), by studying the interactions between sodium dodecyl sulfate (SDS) and mouse interferon in an attempt to gain further insight into the structure of interferon; and (iii), by exploring more efficient and effective ways of purifying mouse interferon.

The first chapter concerns the structural and functional similarities between interferons of different species. Antibodies directed against mouse interferon, human leukocyte interferon and human fibroblast interferon were used in a cross-species antibody interferon-neutralization assay as a means of identifying common antigenic sites amongst the three types of interferon. By testing the effects of interferon-neutralization on both homologous and heterologous cells, one may obtain some insight into the similarities between the active sites of the three forms of interferon and their interactions with interferon cell receptors.

Interferons also appear to be relatively unique in that not only do they remain biologically active when treated with the denaturant sodium dodecyl sulfate (SDS), but the presence of the detergent also stabilizes interferons to a variety of other denaturants. Since SDS has an apparent special relationship with interferon, SDS was used as a probe in an attempt to gain further knowledge of the chemical structure of interferons. In the second chapter, analogs of SDS, varying in size and composition, were employed to investigate both the mechanism of interferon stabilization by SDS and the physicochemical requirements of the detergent involved in this

stabilization process. The interactions between SDS and interferon were examined by ionic exchange chromatography, hydrophobic chromatography and Controlled Pore Glass (CPG)-adsorption chromatography.

A complete elucidation of interferon's structure-function relationship may only occur if the protein is pure enough, so that both its primary and tertiary structures can be determined. Chapter three describes several purification techniques which may further that goal. Two concert chromatographic schemes were developed. With both procedures, the conditions for adsorbing and desorbing interferon were coordinated so as to reduce the amount of time and the number of manipulations required for purification and thus diminish the possibility of inactivation. The first scheme employed hydrophobic chromatography with AFFI-Gel 202, and CPG-adsorption chromatography. The second scheme involved gel filtration chromatography with Ultrogel AcA 54, ionic exchange chromatography with CM-Bio-Gel Agarose, followed by the procedures employed in the first scheme.

CHAPTER ONE

IMMUNOCHEMICAL CHARACTERIZATION OF MOUSE INTERFERON

INTRODUCTION

Antibody Production in Goats to Mouse L-929 Interferon

Antibodies directed against interferons have been raised in an assortment of animals and under various regimens. Most notable were antibodies to mouse interferon produced in guinea pigs (97), rabbits (75,94), sheep (49, 112), and goats (113), and to human interferons in guinea pigs (89), rabbits (18,89), and sheep (4,89). Depending upon the size of the animal, doses contained as little as 1.5×10^3 units in guinea pigs (97), and 6×10^4 units in goats (113), to as much as 2.4×10^6 units (89), and 3×10^7 units (89), in guinea pigs and sheep respectively. The neutralization titer obtained depended primarily upon the amount of interferon invested as an immunogen. Reported titers were as low as 1.5×10^2 neutralizing units (one unit being defined as the amount needed to neutralize the antiviral activity of one unit of interferon), in guinea pigs (97), to as high as 2.4×10^6 in sheep (49).

In reports employing large animals, where the greatest quantity of serum can readily be obtained, the rate of antibody synthesis with respect to the amount of interferon injected is modest. The approach applied in this report was to improve the immunogen environment and thus to enhance the rate of anti-interferon production without a concomitant increase in the amount of interferon invested.

Seeking a more efficient immunization procedure, two techniques to enhance interferons' in-vivo stability and immunogenicity were investigated. Rather than injecting the material in an aqueous solution or oil/water emulsion, as previously described (4,10,75,89,94,97,112,113), preparations were lyophilized and reconstituted into oil only (Freund's adjuvant). Thus the physiological milieu of the animal served as the aqueous phase, while the protein was contained within the oil. "Anhydrous oil vaccines", such as this, have been previously reported (55) for use with labile proteins which, under other conditions, are rapidly denatured.

The cross-linking of the immunogen to itself and to a carrier of larger molecular weight (human serum albumin) by glutaraldehyde was also examined. The former approach has proven succesful in raising antibody to calf

thymus terminal deoxynucleotidyl transferase (17). The latter approach, where a large molecular weight carrier is employed, has been applied for many haptens (6,103).

Both methods gave detectable quantities of anti-interferon within nine to eleven weeks, and following further injections, high antibody titers were obtained with respect to the amount of interferon invested.

The Dissociation and Separation of Interferon from Anti-Interferon

Evidence of the existence of antibodies directed against interferons is based upon their ability to neutralize antiviral activity. However, attempts to utilize these antibodies as a means of identification in immunoprecipitations (106), Ouchterlony immunodiffusion (98), and rocked immunoelectrophoresis (45), have yielded dubious results. That interferon can be found within these precipitants and that it remains active has yet to be determined. An ideal resolution of these questions would be realized from dissociation of the antigen-antibody complex, separation of the components, and recovery of biologically active material. Such a technique could also be employed to identify precipitants containing interferon, among others in a complex antigen-antibody system, which could then be cut out to serve as a more purified immunogen (28,70,73,116).

A multitute of procedures have been described for the dissociation and separation of complexes. A few of the more applicable are such non-specific treatments as acid (64,65,66,134), alkali (66), aqueous carbon dioxide (134), freeze-thawing (105), and denaturants (66).

As interferon is extremely stable to low pH treatment, this seemed the most promising. Under conditions of low pH and moderate ionic strength, interferon-anti-interferon products can be dissociated, rapidly separated, and the antiviral activity restored. This procedure is applicable to both aqueous samples and immunoprecipitants.

Neutralization of Interferon Activity in Homologous and Heterologous Cells with Homologous and Heterologous Antibody

Based on early reports examining the heterospecific activity of interferons (7,83), many investigators included "species specificity" among the criterion for identifying interferon. Conflicting evidence suggested however, that uncharacterized interferon was active, usually to a lesser (19,54,111), but sometimes to a greater extent (24,58), on cells of another species.

Employing better defined preparations, interferons of one species have been shown to protect cells of another species, to a diminished degree as compared to their native interferon, in the following combinations : human leukocyte interferon on bovine, porcine and murine cells (100); mouse interferon on human cells (12); avian interferons on other avian cells (87); and bovine interferon on porcine (2), monkey and human cells (132). Moreover, some interferons, of heterologous origin, have been reported to have more activity than homologous interferons. Examples of this are human fibroblast interferon on rabbit cells (40), human leukocyte on bovine cells (48), bovine interferon on lamb cells (108), and the low molecular weight component of human leukocyte interferon treated with sodium dodecyl sulfate, under reducing conditions, on feline cells (39).

Two principle articles have offered an original means of studying the cross-species specificity of interferons with respect to heterologous antibody. The first (74) describes the effects of antibodies, raised against rabbit and mouse interferons, on the priming effect and protection of human interferons. The studies were then extented in a second article (99) and a model was proposed suggesting that human interferons contain multiple reactive sites, and that each molecule is capable of interacting with cells of different species.

Although previously only suggested (74,99), definitive evidence is now presented that human fibroblast interferon is capable of protecting mouse cells against viral infection, and when compared to mouse and human leukocyte interferon, it is about twelve-hundred, or twenty times less active, respectively.

Based on the above relationship, and the previous knowledge of mouse

interferon's ability to protect human cells (12), the neutralization of human and mouse interferons by both homologous and heterologous antisera was examined on both homologous and heterologous cells. The interactions between interferon antibody, and the cell receptor are discussed, and two additional models regarding the mechanism of cross-species specificity are considered.

MATERIALS AND METHODS

Cells and Viruses

L-929 cells, and human diploid skin fibroblasts (VGS strain) were grown in Eagle's Minimal Essential Medium (EMEM) supplemented with ten percent fetal calf serum (FCS). The Kumarov strain of Newcastle Disease Virus (NDV) was used for interferon induction. The stock virus was prepared in the allantoic fluid of ten-day-old chick embryos. The challenge virus used for all assays was the Indiana strain of Vesicular Stomatitis Virus (VSV). The stock virus was propagated in BSC-1 cells.

Interferons

Crude mouse interferon (MIF), was produced by a modification of the procedure described by Knight (67). L-929 cells, grown to near confluency in 1/2 gallon roller bottles, were primed for 2 hr, at 37°C, with 100 units/ml of MIF in EMEM containing 10 % FCS. The primer was decanted and replaced with 1.4×10^5 pfu/ml of NDV in EMEM. After incubation for 1 hr at 37°C, the NDV was discarded, and the cells washed in phosphate buffered saline (PBS), containing magnesium and calcium. The bottles were then replenished with EMEM and incubated at 37°C, for 18 hr.

Human diploid fibroblast interferon (HFIF), and human leukocyte interferon (HLIF) were the generous gifts of Dr. V.G. Edy, Leuven, Belgium and Dr. Kari Cantell, Helsinki, Finland, respectively.

Interferon Assays

Mouse interferon was assayed by the inhibition of VSV-induced cytopathogenicity (C.P.E.) in L-929 cells. All assays included an internal standard calibrated against the National Institute of Health mouse reference standard G 002-904-511.

Both HLIF and HFIF were titrated in a similar VSV C.P.E. inhibition assay, but on VGS cells. All assays included an internal standard calibra-

ted against the British Research Standard for Interferon MRC B69/19.

In all assays except those of VGS cells treated with MIF, heterologous activity was detected by VSV C.P.E.-inhibition.

To titrate MIF on VGS cells, a virus yield-reduction assay was employed. Human diploid fibroblasts, grown to confluency in 60 x 15 mm tissue culture dishes (Falcon), were incubated with MIF for 18 hr at 37°C. Cells were then washed three times with EMEM and challenged with 1.2×10^4 pfu/ml VSV. After 1 hr at 37°C, the cells were washed once in EMEM, and incubated for a further 18 hr at 37°C, in EMEM containing 3 % FCS and 1 % sodium bicarbonate. Cells were then frozen at -70°C, thawed, scraped, and progeny titrated in a plaque assay using L-929 cells.

Anti-Interferon Neutralization Assay

The neutralization endpoints of antibodies directed against interferons were determined by a modification of the microtiter technique described by Havell et al. (52). Prior to incubation with cells, antibody (Ab)-antigen (Ag) mixtures were incubated for 4 hr at 37°C. The titers were expressed as the highest two-fold dilution which restored partial antiviral activity (i.e. first indication of protection), multiplied by the final concentration of interferon used.

When tested on VGS cells, anti-MIF endpoints, as well as anti-HFIF and anti-HLIF mixed with MIF, were determined in a neutralization assay, based on virus yield reduction as described above.

Cross-Linking of Mouse Interferon to Itself and to Human Plasma Proteins

MIF was cross-linked to itself, by a modification of the technique described by Bollum (17). To 14 ml of PBS containing 5.0×10^5 units/ml of MIF, was added 0.14 ml of 10 % glutaraldehyde, in PBS, (Merck, electron microscopy grade). The reaction was allowed to occur for 20 min at 20°C, and was then terminated with 0.7 ml of 1 M sodium borohydride, in PBS.

Mouse interferon was also coupled to human plasma protein, (HPP) which contains 85 % human serum albumin, (National Blood Transfusion Ser-

vice, Belgian Red Cross), by allowing 7 ml HPP (0.4 mg/ml), 7 ml MIF (containing 1.0×10^6 units/ml in PBS, and 0.45 mg/ml protein) and 0.14 ml 10 % glutaraldehyde to react for 30 min at 20°C. The reaction was then terminated with 0.7 ml 1 % sodium borohydride in PBS.

The combination MIF-MIF and MIF-HPP, referred to in the text as treated MIF, were pooled, and divided into thirteen 2 ml aliquots and stored at 4°C. Each dose, used for immunization contained 5.4×10^5 units and 0.65 mg protein.

Immunization

Two year old, female goats, approximately 20-26 kg in weight, were immunized. The immunogen was ammonium precipitated MIF (67), which had an approximate specific activity of 1.0×10^6 units/mg protein.

Subcutaneous injections of treated MIF (i.e. coupled to itself and HPP) were made weekly. In the first three injections the MIF was emulsified in 2 ml Freund's complete adjuvant (Difco), and in incomplete adjuvant for subsequent injections. Occasional boosters, using both complete and incomplete adjuvant, were administered.

Untreated MIF was lyophilized, and the powder pulverized into 4 ml Freund's incomplete adjuvant for the first eleven weeks, into Freund's complete adjuvant in the 12th week, and into either for subsequent boosters. Each subcutaneous administration contained approximately 1.0×10^7 units and 5-10 mg protein.

Bleeding of Goats, Serum Preparation, and Gamma Globulin Fractionation of Serum

Goat were bled from the jugular vein with a 17-19 gauge needle. Blood was withdrawn into sterile glass containers and, to promote clotting, incubated at 37°C for 3 hr. The clot was pelleled, at 1.4×10^3 g for 10 min, and the serum removed. The serum was then decomplemented at 56°C for 30 min, and stored at -20°C, 20 ml aliquots, containing 0.1 % sodium azide.

The gamma globulin fraction to goat serum was isolated as essentially

described by Campbell et al. (21). Two modifications were made : solid, rather than saturated liquid ammonium sulfate was used, and the final pellet was resuspended in PBS.

Antigen-Antibody Dissociation and Separation

Equal volumes of MIF and anti-MIF were mixed in a plastic falcon tube and incubated at 37°C, for 4 hr. The mixture was then dialyzed against 1 liter of 50 mM glycine-HCl pH 2.0, for 18 hr, at room temperature. The retentate was then loaded onto a pre-soaked CF-50 Centriflo Membrane Ultrafilter (Amicon), and centrifuged at 1.4×10^3 g for 10 min at 4°C. The filtrate was collected, dialyzed against PBS, and assayed for antiviral activity.

Indirect Immunoprecipitation of Mouse Interferon

MIF and anti-MIF were mixed and incubated as described above. Following incubation, anti-goat gamma G, prepared in rabbits, RAG, (Miles Laboratories), was added so that the protein content of RAG was slightly greater than that of anti-MIF, thus the precipitant reaction was slightly in antibody excess. After incubation, the precipitate was pelleted at 2.1×10^3 g for 10 min at 4°C. Both the supernatant and pellet, resuspended in PBS, were dissociated and separated as described above.

RESULTS

Antibody Production in Goats to Mouse L-929 Interferon

Both animals injected recognized mouse interferon as an immunogen (Figure 1). Although both responses were linear, goat A, injected with 1.0×10^7 units per week of lyophilized interferon, acquired detectable activity three weeks prior to goat M, injected with 5.4×10^5 units per week of treated interferon. In addition, the formers initial titer was ten times greater. For both goats, the highest antibody titer obtained was as follows : goat A, after 15 weeks, had a titer of 5.2×10^5 neutralizing units/ml; while, after 17 weeks, goat M, had 3.2×10^4 neutralizing units/ml.

Goat A's response to interferon was made in the presence of Freund's incomplete adjuvant. Due to concern that further use of this adjuvant would not appreciably enhance the antibody production, subsequent boosters were prepared in Freund's complete adjuvant. As the period between injections was short, it may simply have been coincidental that a raise in titer was then observed.

Initial doses into goat M were mixed with Freund's complete adjuvant. Unfortunately, at the time of the third administration, abscesses at the injection sites were observed, and thus the immediate subsequent injections were in incomplete adjuvant. Although first detection of neutralizing anti-sera was during the period when incomplete adjuvant was administered, it is difficult to assess its role. Even though the titers' increasing trend had already been initiated, it is interesting that subsequent to the twelfth and fourteenth injections, containing complete adjuvant, substantial increases in antibody titers were detected.

With both animals, decreases in titers were found if the weekly regimen of injections was not sustained. An earlier report (89), suggested that one high antibody titers are obtained, a plateau is maintained, for several weeks, even without subsequent boosters. Perhaps, due to the early high response achieved, under our conditions, this plateau was not reached.

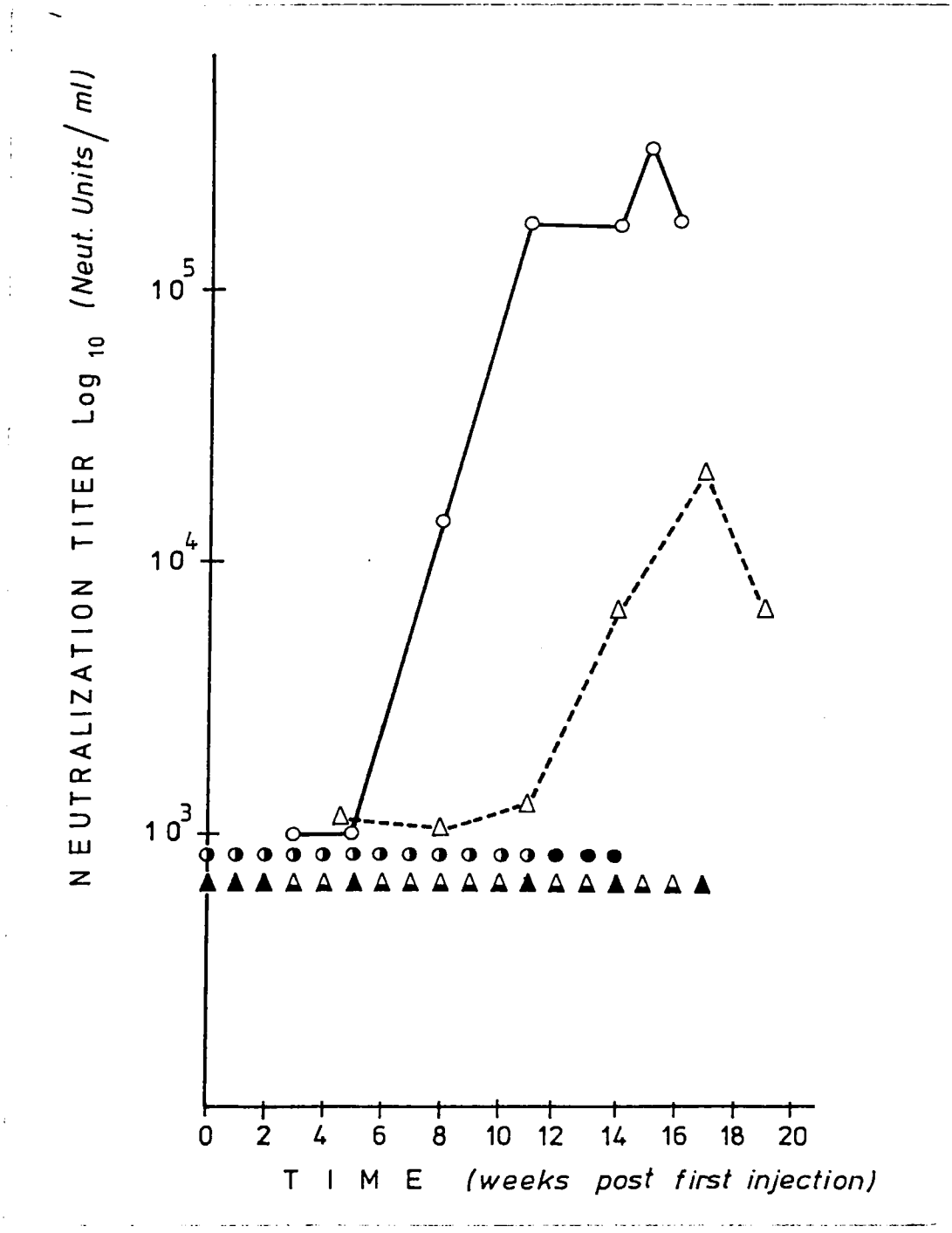

FIGURE 1. Goat A (solid line), was injected weekly with 1.0×10^7 units of untreated MIF, lyophilized, and then pulverized in either Freund's complete (closed circles), or incomplete (half-closed circles) adjuvant. Open circles represent time of bleeding and titer.

Goat B (broken line), was injected weekly with 5.4×10^5 units (in aqueous form) of treated MIF, mixed with either Freund's complete (closed triangles), or incomplete (half-closed triangles) adjuvant. Open triangles represent time of bleeding and titer.

The Dissociation and Separation of Mouse Interferon from Anti-Mouse Interferon

As indicated in Table 1, neutralized mouse interferon can qualitatively be dissociated, separated and detected. Starting material, containing 2.5×10^5 units of interferon, is completely neutralized when treated with 2.0×10^5 neutralizing units of anti-MIF for 4 hr at 37°C. However, when the reactants are dialyzed against 50 mM glycine-HCl buffer, pH 2.0, and centrifuged in a Centriflo membrane, the filtrate contained 6.3×10^3 units of activity. This represents a 2.5 % recovery of the starting material.

Two possibilities exist for the low filtrate yield : that the antigen-antibody dissociation was incomplete, or that the majority of the activity remains on the filter. Filtrates (5.0×10^2 units) of starting material (1.0×10^5 units) which had not been treated with antibody or acid, indicate that the latter is the limiting factor.

To disprove the possibility of Centriflo separation alone yielding detectable filtrate activity, a filtrate from non-acid-treated, neutralized material was also assayed. From the initial 4.0×10^4 units loaded, no activity in the filtrate was detected.

Table 2 presents data on the recovery of antiviral activity from indirect immunoprecipitants. Approximately 1.25×10^5 units of MIF is incubated with 2.0×10^5 neutralizing units (containing 17 mg protein) of anti-MIF, for 4 hr at 37°C. Anti-sera, raised in rabbits, against goat gamma G (RAG), containing 18.4 mg antibody, is added, and incubated for 2 hr at 37°C, followed by an additional 18 hr at 4°C. The immunoprecipitant formed is then pelleted, and both the supernatant and pellet treated as described above. The pellet contained the only detectable activity and represents 0.08 % (1.0×10^2 units) of the starting material.

TABLE 1. Dissociation and Separation of Interferon from Anti-Interferon

Description	Titer (log_{10})
Starting material	5.4
Post-neutralization[a]	< 1.0
Filtrate, post-neutralization and acid treatment[b]	3.8
Filtrate, post-neutralization[c]	< 1.0
Filtrate from starting material[d]	2.7

(a) The titer of the antisera was 2.0×10^5 neutralizing units/ml

(b) Filtrates from neutralized material (containing 2.5×10^5 units) which had been acid treated and passed through a Centriflo membrane.

(c) Filtrates from neutralized MIF (containing 4×10^4 units) passed through a Centriflo membrane.

(d) Filtrates from MIF (containing 1×10^5 units) passed directly through a Centriflo membrane.

TABLE 2. Indirect Immunoprecipitation of Mouse L-929 Interferon

Description	Titer (log_{10})
Starting material	5.1
Post-neutralization[a]	< 1.5
Post-neutralization + RAG[b]	< 2.4
Filtrate from pellet[c]	2.0
Filtrate from supernatant[d]	< 1.8

(a) The titer of the antiserum was 2.0×10^5 neutralizing units.

(b) Supernatant from the neutralized material treated with RAG.

(c) Filtrate from the acid treated pelleted immunoprecipitate.

(d) Filtrate from the acid treated supernatant of the immunoprecipitate.

Neutralization of Interferon Activity in Homologous and Heterologous Cells with Homologous and Heterologous Antibody

A comparison of the activities of mouse (MIF), human fibroblast (HFIF), and leukocyte (HLIF) interferon's activities on heterologous cells

Table 3 presents a comparison of mouse and human interferon's antiviral activities on heterologous cells. Although HFIF is active on L-929 cells, it is approximately twenty-times less so than HLIF. Thus, with respect to the mouse cell, MIF provides greater protection than HLIF, which in turn is more active than HFIF. Similar to what has been previously reported (12), MIF protects VGS cells against viral infection.

Neutralization and cross-species neutralization of MIF, HFIF, and HLIF on homologous and heterologous cells

Titers representing the neutralization and cross-species neutralization of MIF, HFIF, and HLIF by anti-MIF, anti-HFIF, and anti-HLIF assayed on mouse L-929 and human VGS cells are shown in Tables 4 and 5, respectively. Although the data in Table 3 suggests that with respect to the cell receptor there exists a closer structural similarity between MIF and HLIF than between MIF and HFIF, anti-MIF (Table 4) does not recognize this distinction.

In order to equate titers expressed on L-929 and VGS cells, a ratio, based on the results presented in Table 3, has been established. One unit/ml of activity on L-929 cells is approximately equivalent to 1.2×10^3 units/ml of HFIF and 6.0×10^1 units/ml of HLIF assayed in VGS cells. Also, 1.0×10^4 units/ml of MIF, assayed on L-929, is equivalent to 1 unit/ml of activity of VGS cells. Thus the neutralization titer is expressed as the product of the highest dilution of antibody, restoring partial protection, times the number of units/ml of interferon used, as expressed on the cells tested (L-929 cells, Table 4 or VGS cells, Table 5).

The expected titer, determined from the actual results, is what the endpoint would supposedly have been if tested in the other cell system (VGS cells, Table 4 or L-929 cells, Table 5). Therefore, for example, based on the ratio of activities for MIF assayed on both L-929 and VGS cells, a titer of 4.0×10^5 neutralizing units/ml for anti-MIF treated with MIF and

TABLE 3. A comparison of Mouse (MIF), Human Fibroblast (HFIF), and Human Leukocyte (HLIF) Interferon's Activities on Heterologous Cells

Interferon	Amounts of interferon added (units/ml)	Titer on heterologous cells
HFIF	1.0×10^6	8.0×10^{2} (a)
HLIF	1.0×10^7	1.6×10^{5} (a)
MIF	1.0×10^4	1.0×10^{0} (b)

(a) Tested on mouse L-929 cells in a C.P.E.-inhibition assay.

(b) Tested on human VGS cells in virus yield reduction plaque assay.

TABLE 4. Cross-Neutralization Assay Tested on Mouse L-929 Cells

Anti-IF	IF	Titer ($\log_{10}$)	Expected titer[a] ($\log_{10}$) on VGS cells
MIF	MIF	5.6	1.6
	HFIF	3.5	6.6
	HLIF	3.1	4.9
HFIF	MIF	< 2.1	< 0.1
	HFIF	< 1.3	< 4.4
	HLIF	< 2.3	< 4.1
HLIF	MIF	< 2.1	< -1.9
	HFIF	3.6	6.7
	HLIF	4.8	6.6

(a) The expected titer is defined as what the endpoint, determined from the actual results, would have been, if tested on VGS cells.

TABLE 5. Cross-Neutralization Assay Tested on Human VGS Cells

Anti-IF	IF	Titer (log_{10})	Expected titer[a] (log_{10}) on L-929 cells
MIF	MIF	3.0	7.0
	HFIF	1.0	-2.1
	HLIF	2.2	0.4
HFIF	MIF	< 0.6	< 4.6
	HFIF	3.8	0.7
	HLIF	< 2.4	< 0.6
HLIF	MIF	2.7	6.7
	HFIF	5.6	2.5
	HLIF	6.0	4.2

(a) The expected titer is defined as what the endpoint, determined from the actual results, would have been, if tested in VGS cells.

tested on L-929 cells, is expected to have a titer of 4.0×10^1 neutralizing units/ml, if assayed on VGS cells.

The deviation of actual from expected results led to a comparison of the relationship between anti-interferon and interferon tested on homologous and heterologous cells. Interferon-anti-interferon mixtures tested on homologous, (with respect to the interferon), cells indicate an expected titer less than what was actually found (i.e. actual titer) on heterologous, (with respect to the interferon), cells. However, that same interferon-anti-interferon reaction tested on heterologous cells, indicates an expected titer greater than was actually found on homologous cells. Furthermore, this divergence was greatest when the antibody was treated with heterologous interferon.

It is also noteworthy, as previously reported (10,51), that anti-HLIF will neutralize HFIF (Table 5), while the converse was not observed. However, results here indicate a greater antigenic similarity between the human interferons, with respect to anti-HLIF, and this similarity is also reflected on L-929 cells (Table 4). Because of anti-HFIF comparitively low titer, a full assessment of its ability to neutralize MIF or HFIF, on either homologous or heterologous cells, was not possible.

DISCUSSION

Antibody Production in Goats to Mouse L-929 Interferon

The initial intent in immunizing goats with mouse interferon was to obtain antisera for analytical purposes, not a study in itself. It was only after achieving such rapid results that an examination of the procedures employed became of interest.

To compare the effectiveness, as an immunogen, of anhydrous (i.e. lyophilized then reconstituted directly in oil) versus treated (i.e. cross-linked, via glutaraldehyde, to either itself or HPP), MIF, is too difficult. Each scheme involves too many variables and combinations to attempt to evaluate the potential of, for example, the use of Freund's complete as opposed to incomplete adjuvant, treated than untreated (lyophilized) interferon, or the number of units injected.

A methodical approach would be the use of smaller animals to test each of the possible considerations. One such experiment would involve examination of the in-vivo stability of material lyophilized and then pulverized in oil. The injected animal would be periodically bled, and its serum tested for antiviral activity with respect to time. Such a test may demonstrate the applicability of the procedure described here in enhancing interferon's clinical potential.

The evaluation of the effectiveness of the immunization procedures of different investigators is complicated by the difficulties of comparing such elements as the degree of immunogen purity, the scheme employed, and the relative immunogenicity of the various interferons. There are, however, three considerations which can be evaluated : the number of neutralizing units/ml obtained per units of interferon invested; the amount of blood sately obtainable from the animal; and the weeks required to achieve the neutralizing titer. From these variables the Immunization Efficiency Index (IEI), can be determined by the following equation :

$$IEI = \frac{\text{neutralizing units per ml}}{\text{units IF invested}} \times \frac{\text{volume blood attainable}}{\text{weeks required}}$$

The greater the IEI, the more efficient the procedure. Allowing for differences in the definition of neutralizing units, and units of interferon for different species, Table 6 represents the IEI of past procedures compared to the two procedures described here.

Both the treated and untreated (but lyophilized) mouse interferon immunization schemes gave a comparatively high IEI. While the procedure of Gresser et al. (49) yielded a higher IEI, it should be noted that the peak titer was obtained in fifty-five weeks, as compared to fifteen and seventeen weeks for goat A and goat M, respectively. Further, and more exhaustive, testing might prove these procedures to be quite efficient.

Table 6 also indicates that when HLIF is the immunogen, it appears that the larger the animal the greater the IEI, even though the ratio of number of neutralizing units/ml per unit of interferon invested remains the same. However, this was not the case for mouse interferon.

If one assumes the purity of mouse interferon to be 2.4×10^9 units/mg protein (37), injections of 4.2 µg per week of untreated, and of 0.23 µg per week of treated interferon was sufficient to generate specific neutralizating antibodies.

The Dissociation and Separation of Mouse Interferon from Anti-Mouse Interferon

This is the first report of the dissociation and separation of interferon from its antibody both in solution and as an immunoprecipitate. Due to the poor recovery involved, this is, at best, a qualitative procedure.

Size was the criterion used to separate mouse interferon, (MW = 2.2×10^4 daltons, 37), from its antibody, (IgG, MW = 1.5×10^5 daltons). Although the Centriflo membrane is assumed to have a molecular weight cut-off of 5×10^4 daltons, chymotrypsinogen, a 2.5×10^4 dalton molecular weight species, is reported (95) to have greater than 70 % retention. Therefore, one could anticipate the filtrate to contain only a small percentage of the total mouse interferon applied onto the membrane.

The even lower recovery from the immunoprecipitate was most likely due to a higher protein content of the solution obstructing the flow through the membrane. The repeated resuspending of the retentate followed by fur-

TABLE 6. A Comparison of the Immunization Efficiency Index, (IEI), among Various Procedures

	Column A = Neutralizing units/ml obtained	Column B = Column A divided by units of IF invested	Column C = Column B multiplied by volume blood attainable	IEI[(a)] = Column C divided by weeks required
MIF/Goat A	5.2×10^{5}	3.6×10^{-3}	3.6×10^{-1}	2.4×10^{-2}
MIF/Goat M	3.2×10^{4}	2.8×10^{-3}	2.8×10^{-1}	1.6×10^{-2}
MIF/Goat[(b)] (113)	5.1×10^{2}	2.8×10^{-4}	2.8×10^{-3}	1.3×10^{-4}
MIF/Sheep (112)	8.0×10^{3}	5.7×10^{-5}	5.7×10^{-3}	1.6×10^{-4}
MIF/Sheep (49)	2.4×10^{6}	6.8×10^{-2}	6.8×10^{0}	1.2×10^{-1}
MIF/Rabbits (94)	3.2×10^{4}	3.5×10^{-4}	1.0×10^{-2}	2.3×10^{-4}
HLIF/Sheep (89)	1.2×10^{6}	7.0×10^{-3}	7.0×10^{-1}	1.1×10^{-2}
HLIF/Rabbits (89)	1.5×10^{5}	2.3×10^{-3}	6.9×10^{-2}	3.8×10^{-3}
HLIF/Guinea Pigs (89)	3.0×10^{4}	3.0×10^{-3}	3.0×10^{-3}	1.3×10^{-4}
HLIF/Mice (30)	2.5×10^{3}	1.9×10^{-3}	1.9×10^{-3}	1.2×10^{-4}

(a) Immunization Efficiency Index : The formula is based on the following considerations : the number of neutralizing units acquired per total amount of interferon invested as an immunogen; the amount of blood safely attainable from the animal (sheep and goats 100 ml, rabbits 30 ml, and guinea pigs and mice 1 ml); the number of weeks required to obtain the titer.

$$\text{IEI} = \frac{\text{Neutralizing units per ml}}{\text{Units IF invested}} \times \frac{\text{Volume blood attainable}}{\text{Weeks required}}$$

(b) The numbers in parenthesis are the citations.

ther filtration did not resolve this difficulty.

Despite its shortcomings, the Centriflo's advantages are its convenience, rapidty, and success. Of the other potential separation techniques, such as gel filtration, sucrose gradients, and polyacrylamide gel electrophoresis (PAGE), only the latter is reported to have respectable yields of MIF from low starting material (18), and thus the potential of separating MIF from anti-MIF. Preliminary attempts to dissociate interferon from its antibody, in the presence of denaturants (122), proved unsuccessful, and thus rendering the subsequent use of PAGE, as a means of separation, unfeasible.

Two other possible suitable separation procedures are micro-membranes and micro-step-elution chromatography. The use of the micro-membrane would reduce the adsorbing surface area, and could be adapted so as to accomodate a membrane with more applicable separation characteristics.

Although a variety of antigen-antibody dissociation techniques are available, interferon's stability to low pH made acid-treatment the most suitable procedure to employ. Alkali buffers, high salt, chaotrophic agents, and denaturants such as potassium thiocyanate have a less stabilizing effect on interferon, and should therefore be less effective in dissociating antigen-antibody complexes.

Neutralization of Interferon Activity in Homologous and Heterologous Cells with Homologous and Heterologous Antibody

Although prior reports (74,99) have alluded to the effects of HFIF on mouse L-929 cells, this is the first evidence to demonstrate its ability to protect against viral infection, and further, that with respect to L-929 cells, HLIF is more active than HFIF. This may reflect a greater structural similarity between the active sites on MIF and HLIF than between MIF and HFIF.

It is interesting that although L-929 cells distinguish between HLIF and HFIF, antibody directed against MIF does not. This implies that the active sites may not be very antigenic, with respect to anti-MIF, which is possible if they were located within pockets of the molecule and thus not accessible to the antibody combining sites.

In this report anti-HLIF was found to neutralize HFIF to a far greater extent than what was previously described (10,51). That a parallel high neutralization endpoint was also observed on heterologous cells (with respect to the interferon), supports this different finding. The discrepancy might be due to either the cell lines and/or the assay employed, or even more likely, different batches of antisera.

Previous investigators (10,51), have observed that anti-HLIF neutralizes HFIF, while the converse, has yet, not been detected. One explanation may be that while the buffy coats, used as the source of HLIF, may contain a small proportion of HFIF, (which may serve as a contaminant immunogen), whereas HFIF, produced from tissue cultures, would contain no HLIF. Thus, although the anti-HLIF employed in all these investigations was from the same source, the antisera may have been a product of different boosts. A recent report, by Dalton et al. (30), in which antibodies directed against purified HLIF did not neutralize HFIF, supports this explanation.

The results concerning the use of anti-HFIF are not definitive. The titer of the available anti-sera was too low to expect any effective cross-species neutralization to occur. However, the results were included merely to indicate, that as anticipated, no neutralization was detected.

An analysis of the available data indicated that, when assayed on homologous cells (with respect to the interferon), the calculated, expected titer was always less than the actual results obtained when assayed on heterologous (with respect to the interferon) cells. But, when initially tested on heterologous cells, the calculated, expected titer was always greater than the actual results obtained on homologous cells. This might be anticipated if the interaction between interferon and cell were strong for the homologous combination, but weak for the heterologous. If the interferon-cell associations were weak, the antibody could more effectively neutralize the interaction. However, when tested on homologous cells, the actual titer obtained was lower than predicted from the heterologous cell results, because the interferon-cell interactions were stronger and therefore less neutralization occurred. Similarly, when initially tested on homologous cells, stronger interferon-receptor interactions were present, and thus less neutralization occurred. But, when tested on heterologous cells, the actual titer obtained was higher than predicted from the homologous cell results, because the interferon-cell interactions present were weaker and

therefore more neutralization occurred.

The neutralization of interferon by its antibody may take place by two mechanisms. Antibody, once having interacted with the antigen, could either sterically hinder the antigen's ability to associate with a cell receptor, or shift its tertiary structure so that it is no longer recognized by the cell.

By either mechanism, a high neutralization endpoint represents an effective antigen-antibody interaction. That the divergence between the expected and actual results is greatest when anti-interferon is treated with an heterologous interferon, may reflect differences in antibody affinities. Slight antigenic variation between the interferons at these sites, would effect antibody affinities. Thus, although the predominant reaction in the two-step mechanism between interferon, antibody, and cell receptor, is the interferon-receptor combination, the interaction is modulated by differences in the antigen-antibody affinities. Consequently, heterologous antibody has a lower affinity than the homologous, regardless of the cells on which it is tested, and thus the ability to neutralize is even less when tested on homologous cells.

The only possible exception to these observations is the case of HLIF treated with anti-HLIF when examined both on L-929 and VGS cells. However, since HLIF demonstrates a relatively high affinity for both cells, and since the antigen-antibody interaction is between homologous pairs, the expected and actual values may be expected to converge.

Paucker and his colleagues (99) have hypothesized a model to describe the mechanism of interferon cross-species activities. They contend that, at least in the case of HFIF and HLIF, interferons possess multiple active sites, and thus each molecule is capable of interacting with cells of another species. Furthermore, the number and distribution of these sites would vary with the source of interferon, and this accounts for the variable activities observed.

There are however two other possible mechanisms by which cross-species activities can occur. The first considers interferons of a given species to be comprised of sub-populations which although physico-chemically similar, contain slight variations within specific regions. This would be analogous to the structure of antibodies. One, or many, of these sub-populations can fully interact with cells of certain other species. But as they comprise

only a fraction of the total population, the degree of protection would be less. Therefore, instead of each molecule containing a different number and distribution of heterologous sites (99), which would be cumbersome for a molecule of this size, multiple sites are attributed to sub-populations.

The other model considers only one interferon within a given species (Figure 2). The active sites between species would be similar and the degree of cell protection conveyed would be dependent upon the physico-chemical fit between interferon and cell receptor. By this arrangement the receptor, in concert with either some amplification (25), and/or activator (26) system, would modulate the cells response to interferon. The closer the fit, the greater the signal conveyed, and the more protection conferred. In addition, this model would also apply for cell hybrids which contain the receptor of one species but the antiviral machinery for another (114).

The evidence in this report support the last model. As one would anticipate when interferon is added to heterologous cells, a weak fit would create both greater neutralization by antibody and a diminished antiviral signal. Conversely, as seen with interferon and homologous cells, less neutralization and a greater antiviral signal would suggest improved interferon-receptor binding. A variation of this variable-fit model is if heterologous interferon binds to a different cell receptor thus generating another signal. Both the Paucker (99) and sub-population (see above) models require the interaction between interferon and cell to be uniform, since differences in heterologous activities is a function of relevant populations, rather than, in response to interferon-receptor affinities, cellular modulation.

As a control measure, antisera adsorbed on cells, had essentially the same titer as with the non-adsorbed antisera (Tables 4 and 5). This eliminates the possibility that the results obtained could be attributed to anti-bodies directed against antigenic determinants of cellular origin.

In support of some of our findings, Gresser et al. (49), reported that anti-HLIF, having a titer of 2.0×10^5 neutralizing units/ml against HLIF on human cells, had a titer of 1×10^1 neutralizing units/ml when assayed with MIF on mouse cells.

A logical sequel to the reported studies would be the cross-species neutralization of interferons which are more active on heterologous cells

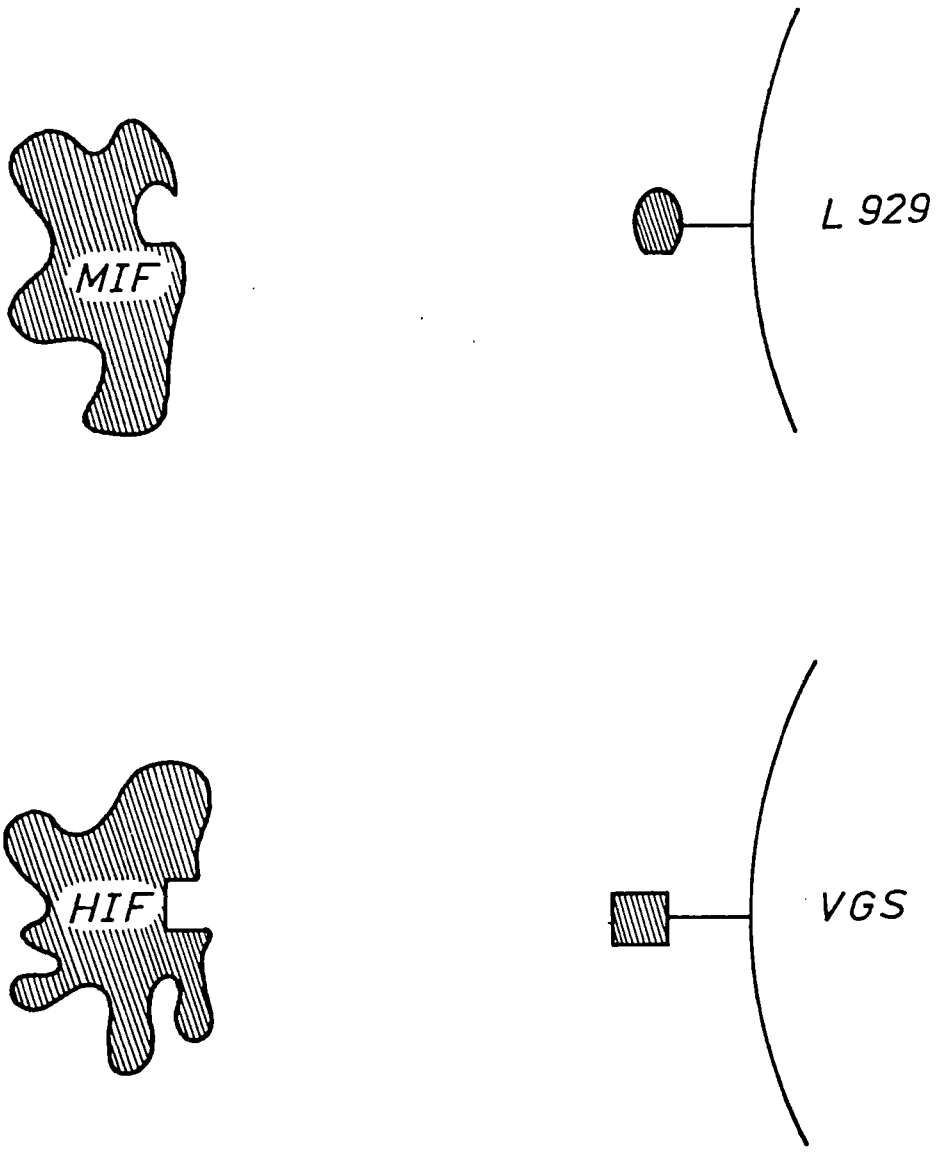

FIGURE 2. This is a diagrammatic representation of the variable fit model. Mouse (MIF), and human (HIF) interferons possess similar active sites and that the degree of protection conveyed is a function of how well these sites fit a cell receptor (human VGS or mouse L-929).

(39,40,48,108), particularly with antisera derived from the same animal as the heterologous cell (99).

It would also be interesting to test anti-interferon's ability to neutralization interferon's other acknowledged biological properties such as cell-growth inhibition (20,37,68,126,133), enhancement of cell cytotoxicity to double-stranded RNA (34,120), and particularly its immunoregulatory effect (47,57,76,117). Perhaps with this approach, a distinction between interferon's multiple functions can be discerned.

SUMMARY

1. Two immunogen preparations were examined for their ability to produce antibodies directed against mouse interferon (MIF). Both were designed to enhance MIF in-vivo stability and immunogenicity. The first, tested in goat A, was lyophilized MIF, reconstituted directly into Freund's adjuvant (anhydrous procedure). Fifteen weeks after the first injection the antisera had a titer of 5.2×10^5 neutralizing units/ml. The other, tested in goat M, was MIF cross-linked, via glutaraldehyde, to either itself or human plasma protein (HPP). Seventeen weeks after the first injection, the antisera had a titer of 3.2×10^4 neutralizing units/ml. Except for one report (49), along with sheep immunized with human leukocyte interferon (HLIF) (89), the Immunization Efficiency Index (IEI), for these two techniques, were superior to previous procedures.

2. MIF could be qualitatively dissociated and separated from anti-MIF, both in solution and as an immunoprecipitate, by dialysis against low pH buffer followed by selective membrane-filtration.

3. Human fibroblast interferon (HFIF) can protect mouse L-929 cells against the cytopathic effect of VSV. However, it was twenty, and twelve-hundred times less active than HLIF and MIF, respectively. This relationship suggests, that with respect to the mouse L-929 receptor, there exists a greater similarity between the active sites of MIF and HLIF than between MIF and HFIF.

4. Employing antibodies directed against MIF, HFIF and HLIF, as a probe, it was shown that interferon-cell interactions were stronger between homologous, as opposed to heterologous combinations. It was hypothesized that a variable-fit model would explain the differences that interferons display in cross-species protection. Thus, the closer the fit between interferon and receptor, the greater the antiviral response.

CHAPTER TWO

SODIUM DODECYL SULFATE-MOUSE INTERFERON INTERACTIONS

INTRODUCTION

Sodium dodecyl sulfate (SDS) is a member of a special group of lipids called detergents. Chemically classified as a Type A amphiphile (53), it is composed of a twelve-membered alkyl hydrophobic tail, and a polar, sulfate head. It is this polar head, forming strong non-covalent bonds with water molecules, which permits SDS high aqueous solubility. These polar interactions more than compensate for the insolubility of the hydrophobic tail, which, by itself, cannot disrupt the hydrogen bonds of water.

If small quantities of soluble amphiphiles are added to water, a portion is dissolved as a monomeric species, while the remainder forms a monolayer at the air-water interface. Depending upon conditions such as ionic strength, pH, and temperature, when the monomer concentration attains a specific level, additional amphiphiles begin to associate into stable colloidal aggregates known as micelles. The concentration required for formation is the critical micelle concentration (CMC). Part of SDS effectiveness as a detergent can be attributed to its relatively high CMC, which allows for the high monomeric concentration required to affect other molecules such as proteins.

Proteins per se, have been shown to bind near equivalent amounts of SDS (107). However, a more detailed study (101) indicates that although most proteins will bind 90 - 100 % of their weight of SDS, sugar and non-disulfide-containing proteins can bind 70 - 100 % and 140 % respectively. In addition, reduction of disulfide-containing proteins will increase SDS binding capacity to 140 %. The exception to the above observations (91), are glucose oxidase, papain and pepsin which do not bind measurable amounts of SDS, and do maintain full enzymatic activity. The general consensus is that SDS has a lower affinity and binding capacity for proteins with a tightly ordered structure, several disulfide bonds and/or subunits.

The association between SDS and proteins is composed of two phenomena. The first involves the hydrophobic interaction between detergent tails and non-polar regions of the proteins; the other is between the amphiphile's sulfate group and positively-charged amino acids such as lysine (80,140). Whether the detergent is located within the protein (140), on its surface (137), or both (140), is dependent upon the extent of protein unfolding and

refolding, and the accessibility of the required regions. At low detergent concentrations, SDS binds to very discrete sites on the protein. The degree of binding is a function of pH, ionic strength, and temperature. Furthermore, when amphiphile and protein are at low concentrations, it is the monomeric species which binds, not the micellar form. Thus, it is the monomer concentration, which governs the amount of detergent that binds to the protein (78,107). Since adding further detergent only increases the size and number of micellar forms, little additional protein-detergent interactions occur.

It is not clear whether SDS has a preference for some amino acids. Some which have been reported to play a role are histidine (110), alanine and proline (79), and phenylalanine, alanine, isoleucine, tryptophan, and threonine (140).

Other amphiphiles such as anionic deoxycholate (78), neutral triton X-100 (78), and cationic tetradecyltrimethylammonium chloride (93), also bind to proteins. Although their protein-binding sites have been described, at the same concentration as SDS,none exhibit, of the cited amphiphiles, the ability to enhance further detergent binding (cooperative binding) and these detergents usually require higher concentrations for protein unwinding to occur. Furthermore, due to their low CMC, the monomeric forms necessary to associate with proteins are usually not sufficiently available.

Most proteins possess a limited number of high affinity binding sites. When free detergent has saturated all of these sites, binding to other sites may occur. This originates from conformational changes within the protein presumably due to the accessibility of previously buried hydrophobic regions (139). Upon saturation of all available sites, proteins either undergo a shift from globular to either random coils (82) or alpha-helical (5,72) conformations. Also, as the number of detergent methylene residues is increased from eight to fourteen, so is its ability to affect protein configurations (72).

Interestingly, in the case of lysozyme (140), upon removal of excess SDS, a refolding of the protein, in a conformation similar but not identical to the native one occurs. The refolded material contained SDS within the protein's surface, sub-surface, and deep hydrophobic core.

An anomaly of SDS protein-disrupting characteristics, is its ability to protect interferon against inactivation by boiling, particularly when

in the presence of urea and 2-mercaptoethanol (121,122,124). This phenomena has been reported for a variety of interferons derived from such species as mouse L-929 (122,123), and human diploid fibroblasts (125). Human leukocyte interferon however became stable to boiling only in the absence of these reagents (88,125). Type II mouse interferon, however, does not maintain its antiviral activity in the presence of SDS (117).

Although SDS has been reported to protect serum albumin (80) and certain enzymes (135) against denaturation by urea, in most instances proteins reverted to a normal configuration upon removal of the denaturants. Furthermore, protein renaturation does not occur in the presence of urea and SDS unless both are subsequently removed (135). For reasons not yet fully understood, SDS-treated interferons sustain their biological activities, even after being boiled in the presence of urea and 2-mercaptoethanol (124). To confuse matters further, in the example of human leukocyte interferon (88), SDS, by itself, does not cause any loss of activity, but does hinder the protein's reoxidation from urea. Also, some interferon components, in the presence of these reagents, lose their activity on homologous cells but maintain their activity on heterologous cells (39). Evidence has also been reported that detergent-reactivated human leukocyte interferons do not fully renature (3), although they remain antigenically indistinguishable from untreated material (9).

Two hypotheses have been suggested for the role of SDS in stabilizing interferons. Stewart and his colleagues (122,123), suggested that SDS protects mouse interferon by unfolding the molecule into a reversible conformation. Upon subsequent removal of SDS, by the presence of serum, the protein renatures. Later on, Allen and Stewart (3), contended that SDS-treated human leukocyte interferon does actually not renature. The latter phenomenon might be attributed to SDS trapped with refolded interferon molecules, which suggests a role for SDS in the refolding process. In our investigations we have attempted to clarify the role of SDS in protecting interferons, from what normally would be drastic denaturing conditions, from three approaches : (i), to define the structural requirements of the detergent necessary to stabilize mouse interferon in the presence of heat, urea, and 2-ME; (ii), employing anion exchange, Controlled Pore Glass (CPG)-adsorption, and hydrophobic chromatography, to determine the physical and chemical interactions between interferons and SDS; and (iii), compare the biolo-

gical activities of detergent-treated and untreated interferon preparations, *via* their susceptibilities to heat and antibody.

MATERIALS AND METHODS

Cells and Virus

Cells and viruses used in the production and assaying of mouse interferon have been described in the previous chapter.

Chemicals

Urea, 3,8-diamino-5-methyl-6-phenylphenanthridinium bromide, di-isobutylphenoxyethoxyethyldimethylbenzylammonium chloride, sodium salts of butyl- (B), octyl- (O), decyl- (D), tetradecyl- (TD), and hexadecyl (HD) sulfate, and 1-octane, 1-dodecane, 1-tetradecane sulfonate were all products of Merck (Darmstadt, Germany).

Anion exchange resins AG1-, X2 (200 - 400 mesh) and X10 (100 - 200 mesh), sodium dodecyl sulfate (SDS or DD), and 2-mercaptoethanol (2-ME), were supplied by Bio-Rad Laboratories (Richmond, California).

Citraconic anhydride was obtained from Koch-Light (Coinbroock Bucks, England), dodecyamine from Fluka AG (Buchs, Switzerland), lithium dodecyl sulfate from Searle (Buchs, England), ^{35}S-SDS (12 mCi/mM) from the Radiochemical Centre (Amersham, England), Dimilume-30 scintillation cocktail from Packard (Groningen, Netherlands), human plasma protein (HPP) from the National Blood Transfusion Service (Belgian Red Cross), controlled pore glass beads (CPG, 10-350B) from the Electro-Nucleonics (Farfield, N.J.) and alkyl-agaroses from Miles Laboratories (Elkart, Indiana).

Interferons

Two different purities of mouse interferon preparations were employed. One, designated AmS/MIF, was from crude material purified to a specific activity of 2.0 - 5.0 x 10^5 units/mg protein, by ammonium sulfate precipitation (67). The other, designated as CPG/MIF, was AmS/MIF, purified further by controlled pore glass (CPG) adsorption chromatography (43) to a specific activity of 5.0 - 10.0 x 10^6 units/mg protein.

Assays

Both interferon and anti-interferon neutralization assays were, as described in the first chapter, by C.P.E.-inhibition.

Treatment of Mouse Interferon with SDS and Other Amphiphiles

Mouse interferon (MIF), was either treated with SDS (SDS-MIF) in the presence of urea, 2-ME and boiled for 10 min, as previously described by Stewart et al. (122), or incubated at 23°C, for 10 min, in the presence of SDS only. Unless specifically stated, SDS-AmS/MIF (3.5 mM final concentration SDS) was prepared by incubation at 100°C, for 10 min, in the presence of 2-ME (14 mM final concentration) and urea (5 M final concentration), while SDS-CPG/MIF (3.5 mM final concentration SDS) was prepared by incubation at 23°C for 10 min (in the absence of urea and 2-ME). ^{35}S-SDS (SDS^{35}), reacted with MIF (SDS^{35}-MIF) was composed of a detergent mixture containing 1 part SDS^{35} to 9 parts SDS.

In order to equilibrate the solubilities of the compounds tested in the "molecular length" (Figure 1) and "other amphiphiles effects" (Table 1) experiments, all amphiphiles were pre-incubated at 60°C. In all experiments, after treatment, samples were dialyzed against either EMEM, containing 10 % FCS, or against PBS. Similar results were obtained regardless of the dialyzing solution used.

Anion Exchange Chromatography

Samples dialyzed against 10 mM Tris-HCl + 50 mM NaCl buffer (Tris-HCl), pH 7.6, were loaded onto columns (0.9 x 2 cm) pre-equilibrated in the same buffer, containing either AG1-X2 or AG1-X10. Columns were then washed in Tris-HCl and 10 - 1 ml fractions were collected. All fractions contained 0.1 ml HPP (22.5 mg/ml) and were apportioned as follows : one-tenth was assayed for antiviral activity; the remaining 0.9 ml were mixed with 10 ml Dimilume and the amount of radioactivity was determined in a Packard scintillation counter (Model 3375).

CPG-Adsorption Chromatography

Columns (0.9 x 2 cm) containing CPG were equilibrated in either Tris-HCl or PBS. Samples, pre-dialyzed against the equilibration buffer, were loaded, and the column subsequently washed in the same buffer. The columns were then washed with 400 mM glycine-HCl buffer (gly-HCl), pH 2.0, and 10 mM Tris-HCl, pH 8.9. Ten - 1 ml fractions of each eluant were collected. All fractions contained 0.1 ml HPP and were apportioned as described above.

Hydrophobic Chromatography on Hexyl-Agarose

Samples, dialyzed against Tris-HCl, were loaded onto columns (0.7 x 5.3 cm) containing hexyl-agarose equilibrated in the same buffer. Columns were then washed in Tris-HCl, and Tris-HCl containing 10 M ethylene glycol + 1 M NaCl (EG-NaCl), and 10 - 1 ml fractions from each wash were collected. All fractions contained 0.1 ml HPP and were apportioned as described above.

Citraconylation of MIF

One ml of MIF, containing 4.0×10^5 units of activity and 1.6 mg protein, was dialyzed against 100 mM sodium potassium buffer (Na-PB), pH 8.0. To this, 3 µl of citraconic anhydride was added, and while vigorously stirring, the pH was maintained at 8.0 with 5 N NaOH, and the mixtures incubated at 23°C, for 4 hr. The material was then dialyzed against 0.1 Na-PB. pH 8.0.

Removal of protein-bound citraconyl groups (CT-MIF) was then obtained by lowering the pH of the sample, with 1 M HCl, to 4.0, and incubating at 39°C, for 4 hr. The pH was then readjusted with 1 N NaOH to 7.2, and the sample dialyzed against PBS.

RESULTS

Interferon Stability versus Molecular Length of Various Sodium Alkyl Sulfates

In order to evaluate the role of SDS hydrophobic tail in stabilizing interferon to heat, urea, and 2-ME, AmS/MIF (3.2×10^5 units/ml), was treated with sodium salts of various alkyl sulfates (3.5 mM final concentration), having molecular lengths ranging from 9.6 Å to 28.1 Å. In one series, mixtures were boiled in the presence of urea and 2-ME for 10 min. To determine what denaturing effects the alkyl sulfates would have alone, the other series was incubated for 23°C, for 10 min, in the absence of urea and 2-ME.

As presented in Figure 1, when compared to boiled and untreated AmS/MIF (4.0×10^3 units/ml), samples containing butyl-, octyl-, and decyl sulfate, incubated at 100°C, for 10 min, had no effect on sustaining interferon's stability to the combination of heat, urea, and 2-ME. Under the same conditions however, samples containing dodecyl-, tetradecyl-, and hexadecyl sulfate, were all effective in maintaining interferon's activity. All alkyl sulfates tested inactivated AmS/MIF (initial titer 3.2×10^5 units/ml) to the same extent when incubated at 23°C, for 10 min, in the absence of urea and 2-ME (Figure 1).

Paradoxically, alkyl sulfates of molecular lengths greater than or equal to dodecyl sulfate will themselves partially inactivate interferons, but offer a stabilizing effect to interferons when exposed to urea, 2-ME, and boiling. Thus, the size of the detergent, or the extent of its hydrophobicity, plays a critical role in the stabilization of interferon to heat, at least in the presence of urea and 2-ME.

Effects of Other Water Soluble Amphiphiles on MIF Stability

In seeking to further elucidate the physicochemical requirements of detergents in stabilizing interferons exposed to heat, urea and 2-ME, other water soluble amphiphiles were tested. Mixtures containing amphiphile (3.5 mM final concentration), AmS/MIF, urea and 2-ME were incubated at 100°C, for 10 min, dialyzed against PBS, and assayed for antiviral activity.

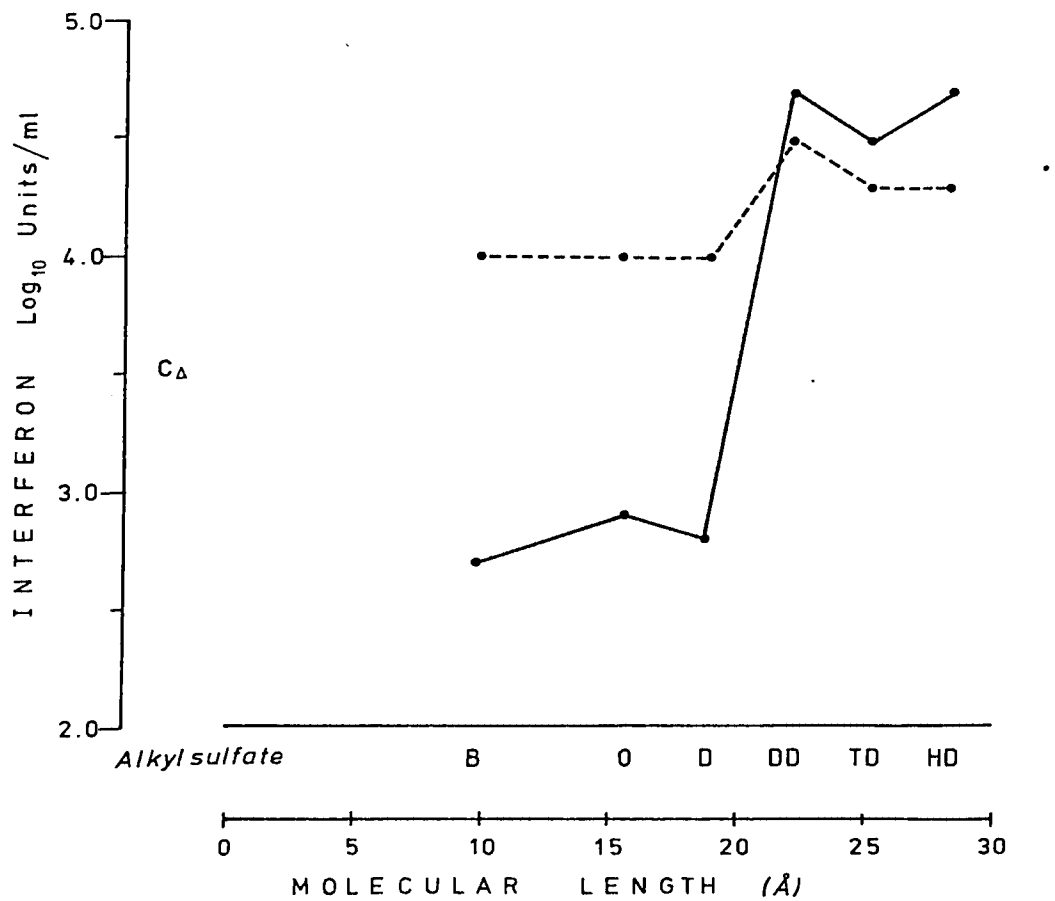

FIGURE 1. Interferon stability versus molecular length of various sodium alkyl sulfates. The solid line represents AmS/MIF-alkyl sulfate mixtures incubated at 100°C for 10 min in the presence of urea and 2-ME. The broken line represents the same mixtures, incubated at 23°C, for 10 min in the absence of urea and 2-ME. In this figure SDS is denoted as DD and boiled material, in the absence of SDS, urea, and 2-ME, as C_{Δ}.

As indicated in Table 1, when compared to SDS, none of the compounds tested, except sodium 1-tetradecane sulfonate, had an appreciable stabilizing effect. Of the amphiphiles dodecylamine, lithium dodecyl sulfate and sodium 1-dodecane, albeit three very similar analogs of SDS, none (except to a slight extent sodium 1-dodecane sulfonate) were active. Although the first two compounds have the same length hydrophobic tails as SDS, their hends contain an amino and sulfonate group, respectively. The third compound, except for its counter-ion, is structurally identical to SDS.

Since it has been reported that in order to acquire the same cooperative binding effect as SDS, some cationic detergents required a 10-fold higher concentration (93), dodecylamine was also tested at a final concentration of 35 mM. The results were the same (Table 1).

Di-iso-butylphenoxy-ethoxyethyldimethylbenzylammonium chloride and 3,8-diamino-5-methyl-6-phenylphenanthridium bromide, were selected because their dominant hydrophobic moieties were aromatic rather than linear in structure. However, as both are also positively-charged species, it is difficult to determine, whether their inactivity was a result of their differences in hydrophobicity and/or charge. That sodium 1-octane sulfonate was inactive could be expected since a similar compound, sodium octyl sulfate, was also inactive (Figure 1).

Comparison of CT-MIF and MIF Stabilities to Boiling in the Presence of SDS

It has been previously reported (80) that SDS stabilizing effects on serum albumin requires the presence of some of the proteins positively charged amino acids, such as lysine. As a consequence of the results obtained with dodecylamine (Table 1), the amino and guanidinium groups of AmS/MIF were reversibly blocked with citraconic anhydride, CT-AmS/MIF, and its stability, after being boiled in the presence of SDS, was compared to non-citraconylated AmS/MIF.

The results presented in Table 2, offer no evidence for the role of lysine or arginine in the stabilization of MIF by SDS. CT-AmS/MIF, boiled in the presence of SDS, and then deblocked, had the same titer as AmS/MIF, which had not been citraconylated, but had been boiled in the presence of SDS. However, boiled CT-AmS/MIF, in the absence of SDS, and then deblocked,

TABLE 1. Effects of Other Water Soluble Amphiphiles on MIF Stability

Compound[(a)]	Titer (log_{10} units/ml)
3,8-diamino-5-methyl-6-phenylphenanthridinium bromide	1.0
di-iso-butylphenoxy-ethoxyethyldimethylbenzyl-ammonium chloride	< 1.0
sodium 1-octane sulfonate	1.7
sodium 1-dodecane sulfonate	2.5
sodium 1-tetradecane sulfonate	3.5
dodecylamine	< 1.0
dodecylamine (35 mM)	< 1.0
lithium dodecyl sulfate	1.5
sodium dodecyl sulfate	4.0
none	1.7

(a) Mixtures of MIF, with the amphiphile, were incubated at 100°C for 10 min in the presence of urea and 2-ME. Unless stated otherwise, all amphiphiles had a final concentration of 3.5 mM, and were pre-heated to 60°C.

TABLE 2. Comparison of CT-MIF and MIF Stabilities to Boiling in the Presence of SDS

Sample Description	Titer (log_{10} units/ml)
AmS/MIF	5.6
CT-AmS/MIF[a]	< 1.5
CT-AmS/MIF, + SDS, 100°C / 10 min[b]	2.2
deblocked AmS/MIF[c]	4.2
AmS/MIF	5.6
CT-AmS/MIF	< 1.5
CT-AmS/MIF, - SDS, 100°C / 10 min	< 1.5
deblocked AmS/MIF	3.2
AmS/MIF	5.6
CT-AmS/MIF	< 1.5
no SDS or 100°C / 10 min	-
deblocked AmS/MIF	4.0
AmS/MIF	5.6
no citraconylation	-
AmS/MIF, + SDS, 100°C / 10 min	4.5
AmS/MIF	5.6
no citraconylation	-
no SDS, but 100°C / 10 min	< 1.5

(a) AmS/MIF which was citraconylated.

(b) CT-AmS/MIF boiled for 10 min in the presence of SDS.

(c) Citraconyl groups removed from CT-AmS/MIF.

was also partially active, but not to the same extent as samples including SDS. This might be possible if citraconylated proteins had already assumed a protective conformation so that the degree of SDS binding and/or the mechanism of renaturation are not fully required.

It is also noteworthy that, prior to being deblocked, CT-AmS/MIF, boiled in the presence of SDS, regained some of its activity, while the sample, boiled without SDS, did not. It is possible, then, that either some CT-AmS/MIF molecules, one complexed with SDS, renatured, or, less likely, SDS was capable of breaking the citraconyl groups' covalent bonds.

Anion Exchange Chromatography of MIF, SDS^{35}-MIF, and SDS^{35}, on AG1-X10

The anion exchanger AG1-X10 has been reported to be an effective means of removing SDS from protein-containing samples (1). By virtue of its high percentage cross-linking (10 %), the effective pore size would exclude large molecular weight molecules such as proteins, while retaining SDS. Thus, since untreated MIF does not bind to the resin, AG1-X10 serves as a useful means of separating free, from protein-bound, SDS.

The results obtained from the anion exchange chromatography of AmS/MIF, SDS^{35}-AmS/MIF and SDS^{35} (Figure 2), indicate that, despite the fact that SDS^{35}-AmS/MIF mixtures had, prior to loading onto AG1-X10, been exhaustively dialyzed, some of the SDS^{35} co-eluted with the MIF, presumably as a complex, while the remainder, presumably in a free state, was bound to the resin.

Approximately 3.2×10^5 units of AmS/MIF were loaded onto a column of AG1-X10. The fractions collected contained a total of 1.6×10^5 units of interferon, or 50 % of the starting material. Fractions collected from SDS^{35}-AmS/MIF (1.6×10^4 units of interferon, and 7.7×10^3 cpm of SDS^{35}), loaded onto a similar column, contained 1.5×10^4 units and 4.2×10^3 cpm, or 93 % and 55 % of the starting material, respectively. However, when 4.7×10^3 cpm of SDS^{35} alone was loaded, there were no counts in any of the fractions collected. Similar results were obtained whether, prior to dialysis with column buffer, the detergent-treated material was dialyzed against serum-containing medium, or PBS. Whether AG1-X10 removes only free or also some SDS bound to the surface of the protein could not be discerned.

After elution of SDS^{35}-AmS/MIF, the exchange resin was removed from

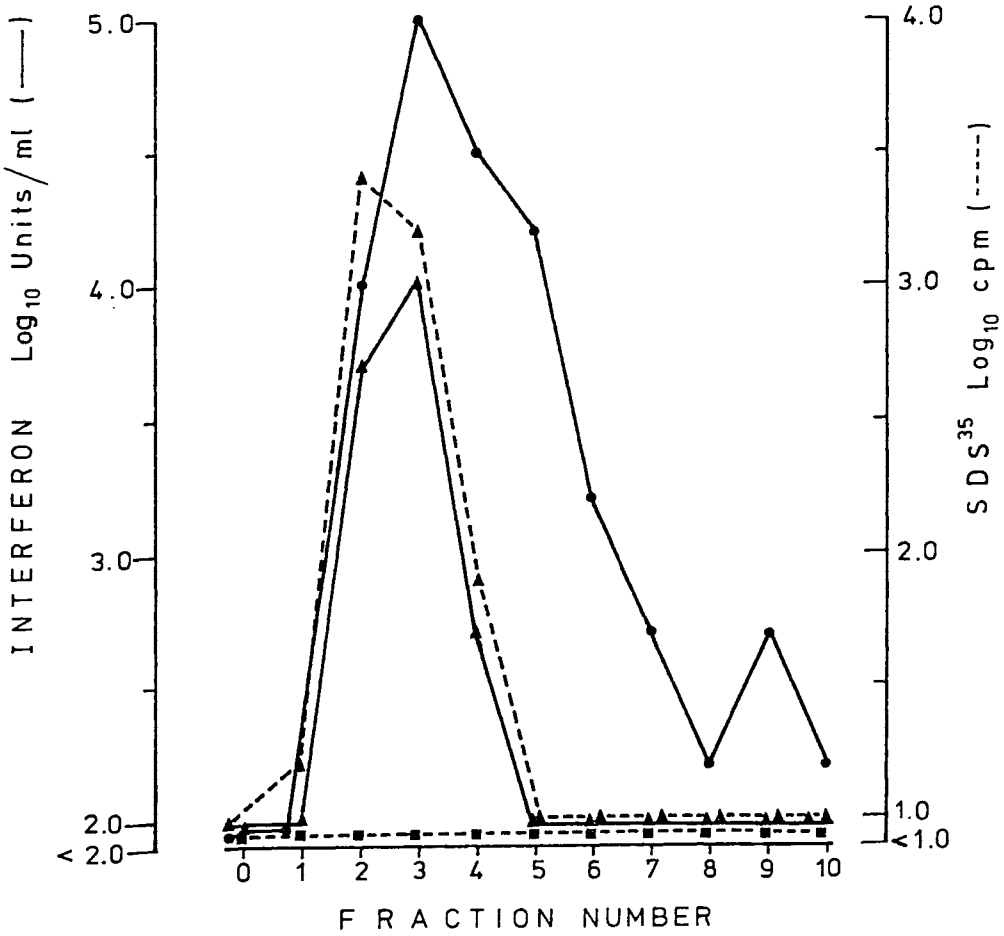

FIGURE 2. Chromatography of AmS/MIF, SDS^{35}-AmS/MIF and SDS^{35}, on AG1-X10. One ml samples of AmS/MIF (circles), SDS^{35}-AmS/MIF (triangles) and SDS^{35} (squares), were loaded onto AG1-X10. Collected fractions were assayed for interferon (solid lines) and SDS^{35} (broken lines).

the column and the amount of SDS^{35} determined. The material contained approximately 1.6×10^3 cpm, or 20 % of the starting material.

In order to demonstrate the resin's capacity for SDS^{35}, 1.27×10^6 cpm, representing 1 mg of SDS^{35}, was loaded onto a similarly-sized column of AG1-X10. The collected material contained 8.6×10^1 cpm or only 0.0067 % of the starting material.

Anion Exchange Chromatography of MIF, SDS^{35}-MIF and SDS^{35}, on AG1-X2

Use of the anion exchanger AG1-X2 has also been reported to remove SDS^{35} from protein-containing samples (135). However, its low percent cross-linkage (2 %) permits higher molecular weight molecules, such as small proteins, to be included within the pores.

As indicated in Figure 3, SDS^{35}-AmS/MIF binds to AG1-X2 while AmS/MIF does not. Approximately, 3.2×10^5 units of AmS/MIF were loaded onto AG1-X2. The fractions collected contained 1.4×10^5 units of interferon or 43 % of the starting material. However, fractions collected from SDS^{35}-AmS/MIF, having 8.0×10^3 units of interferon and 8.7×10^3 cpm of SDS^{35}, contained no detectable interferon or counts. Similarly, fractions from a column loaded with 5.0×10^3 cpm of SDS^{35} did not contain detectable amounts of radioactivity. These results seem to indicate that where it normally would not occur, the binding of interferon to AG1-X2 is achieved in the presence of SDS.

Adsorption Chromatography of SDS-Treated and Untreated Mouse Interferon on CPG

Due to the existence of specific regions containing basic amino acids, and to some extent, hydrophobic sites, mouse interferon binds to beads of CPG (43, and results shown in Chapter 3). To determine whether detergent, bound to mouse interferon, could hinder or mask these sites, the elution characteristics on CPG, of MIF treated with SDS, were examined.

As indicated in Figure 4, while AmS/MIF bound to CPG, SDS^{35}-AmS/MIF did not. Approximately 3.8×10^4 units of AmS/MIF were loaded onto CPG. Fractions collected from the Tris-HCl wash contained no detectable activity,

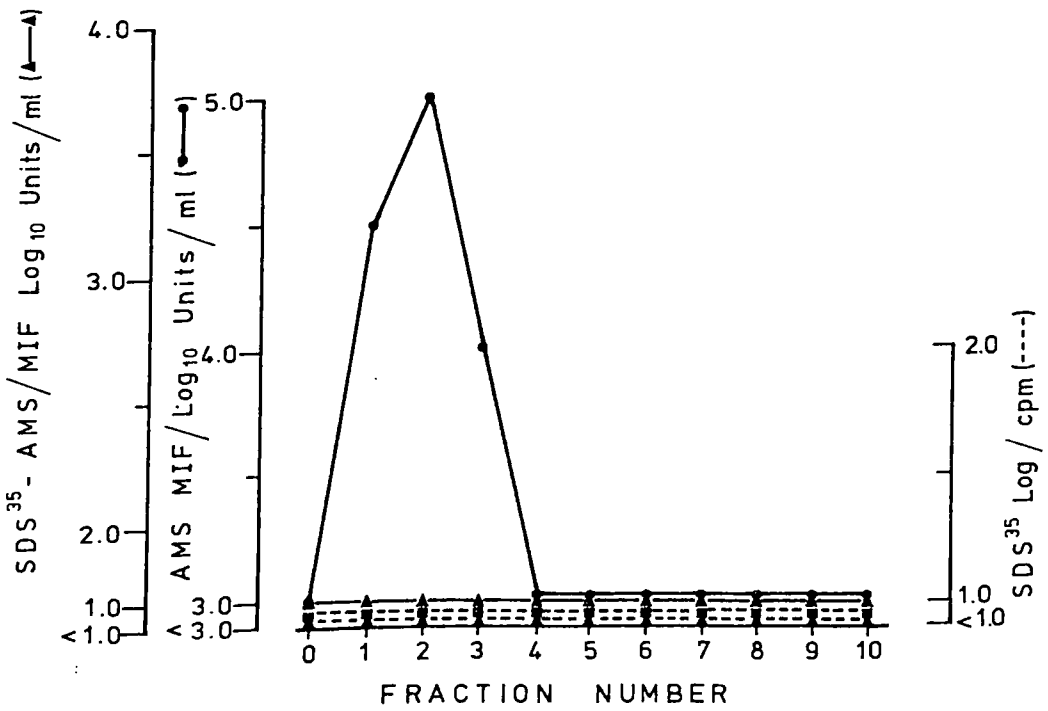

FIGURE 3. Chromatography of AmS/MIF, SDS^{35}-AmS/MIF and SDS^{35}, on AG1-X2. One ml samples of AmS/MIF (●——●), SDS^{35}-AmS/MIF (▲——▲), and SDS^{35} (■——■), were loaded onto AG1-X2. Collected fractions were assayed for interferon (solid lines) and SDS^{35} (broken lines).

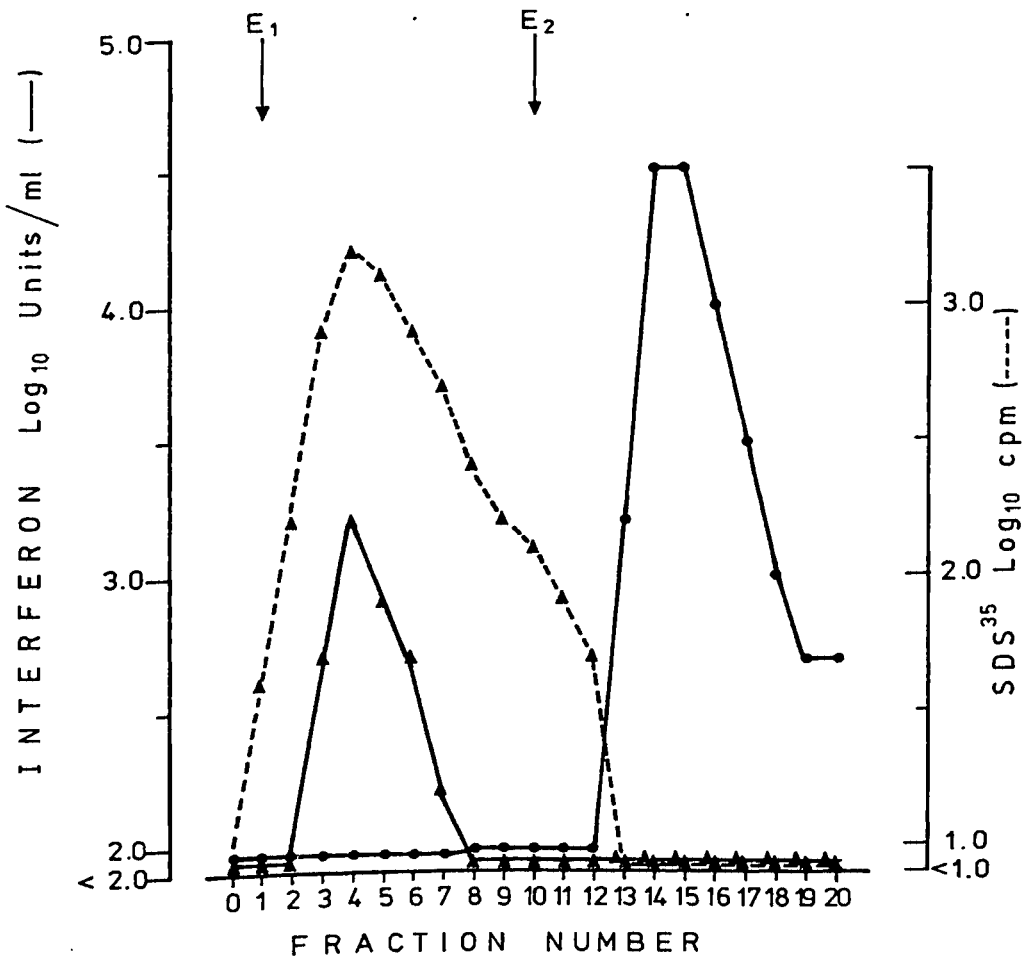

FIGURE 4. Chromatography of AmS/MIF and SDS^{35}-AmS/MIF on CPG. One ml samples of AmS/MIF (circles) and SDS^{35}-AmS/MIF (triangles), were loaded onto CPG. Collected fractions were assayed for interferon (solid lines) and SDS^{35} (broken line). The eluant PBS is denoted as E_1 and 400 mM gly-HCl, pH 2.0, as E_2.

while those obtained from the acid wash contained a total of 6.4×10^4 units of activity, or 168 % of the starting material.

Fractions collected from Tris-HCl wash of SDS^{35}-AmS/MIF (6.5×10^3 units of interferon, and 1.65×10^4 cpm of SDS^{35}), contained a total of 3.5×10^3 units and 8.2×10^3 cpm, or 53 % and 49 % of the starting material, respectively. The acid-wash fractions contained no detectable antiviral activity and only residual counts of SDS^{35}.

Other proteins present in AmS/MIF samples also bind to CPG, but elute under alkali conditions (Chapter 3). After the acid wash, the column, loaded with SDS^{35}-AmS/MIF, was equilibrated in PBS and then washed with 10 mM Tris-HCl buffer, pH 8.5. The collected material contained only trace amounts of interferon (approximately 1 %) but 6.6×10^3 cpm of SDS^{35}, or 40 % of the starting material.

Based on the number of counts of SDS^{35}, it was determined that of the initial 3.5 mM SDS added to AmS/MIF, after dialysis, only 0.026 % (9.2×10^{-3} mM SDS), remained in the loading material. However, since it is known that 35 mM SDS will effectively remove all materials from CPG (96), it was of interest to determine what concentration of SDS would elute MIF from CPG. AmS/MIF (1.0×10^5 units) was mixed with 3.5×10^{-1} mM, 3.5×10^{-2} mM, and 3.5×10^{-3} mM of SDS. Samples were then incubated at 23°C for 18 hr, and loaded onto columns of CPG. As presented in Figure 5, only AmS/MIF treated with 3.5×10^{-1} mM SDS was recovered in the drop-through fractions. It was concluded then, that, since SDS^{35}-AmS/MIF employed in the experiments presented in Figure 4, did not contain a sufficient concentration of SDS to non-specifically desorb AmS/MIF from CPG, that the detergent effects on the protein, as noted in Figure 4, must be quite specific.

As indicated in Figure 6, mouse interferon obtained from the acid wash of a CPG column, and treated with SDS, still did not bind when rechromatographed. Fractions collected from SDS^{35}-CPG/MIF (8.0×10^4 units of interferon, and 3.0×10^3 cpm of SDS^{35}) contained a total of 2.5×10^4 units and 1.5×10^3 cpm, or 32 % and 50 % of the starting material, respectively. The acid wash fractions contained no detectable activity and only residual counts. The subsequent alkali wash had only 0.4 % (3.2×10^2 units) of the input material's antiviral activity and 11 % (3.3×10^2 cpm) of the SDS^{35}.

Similar to SDS^{35}-AmS/MIF (Figure 4), SDS^{35}-CPG/MIF had 6.9×10^{-3} mM of SDS remaining after dialysis. Therefore, even with more purified mate-

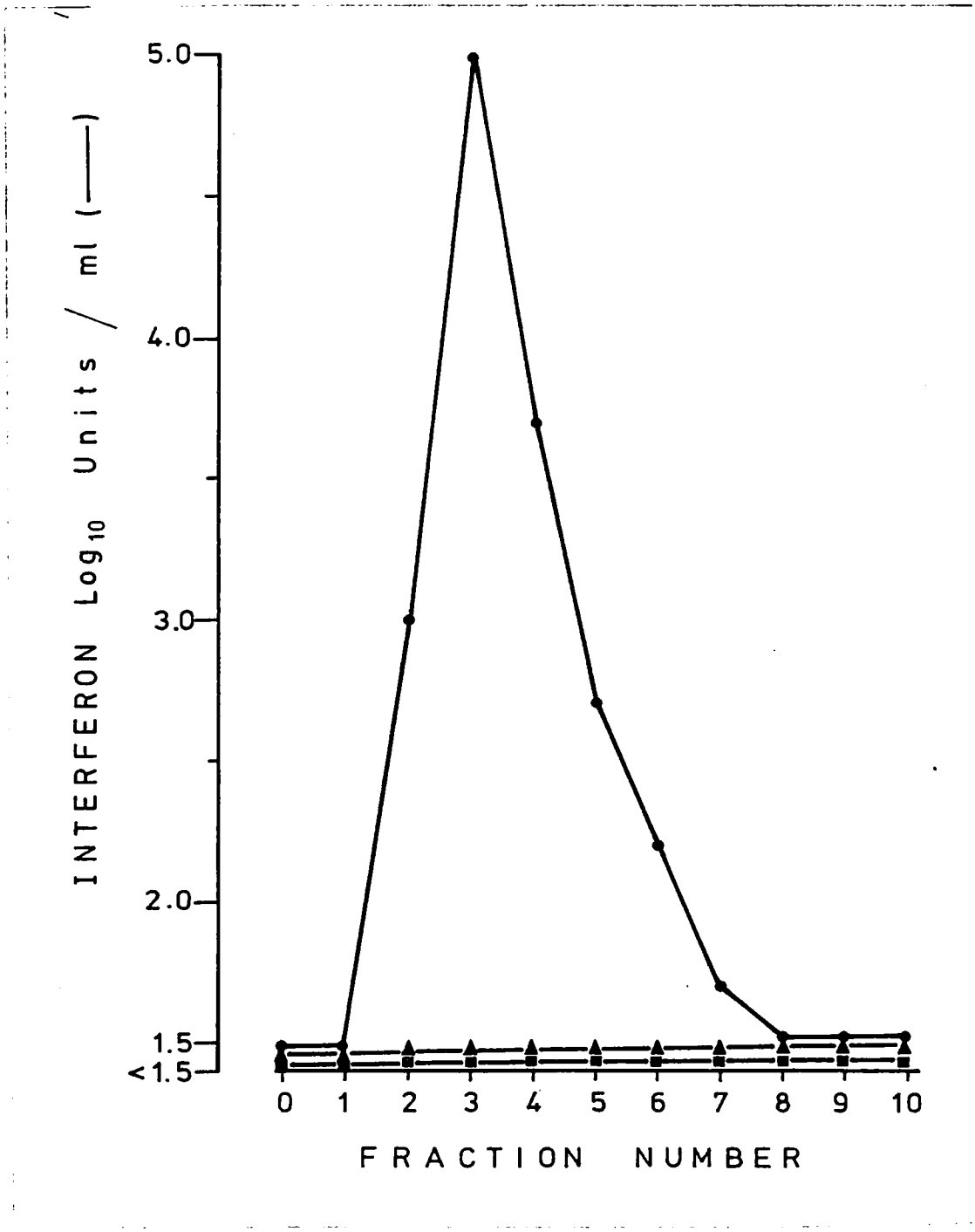

FIGURE 5. CPG chromatography of mixtures of AmS/MIF with different concentrations of SDS. One ml samples of AmS/MIF (1.0×10^5 units) were added to 3.5×10^{-1} mM (circles), 3.5×10^{-2} mM (triangles) and 3.5×10^{-3} mM (squares) SDS. The mixtures were incubated at 23°C, for 18 hr, and then chromatographed on CPG.

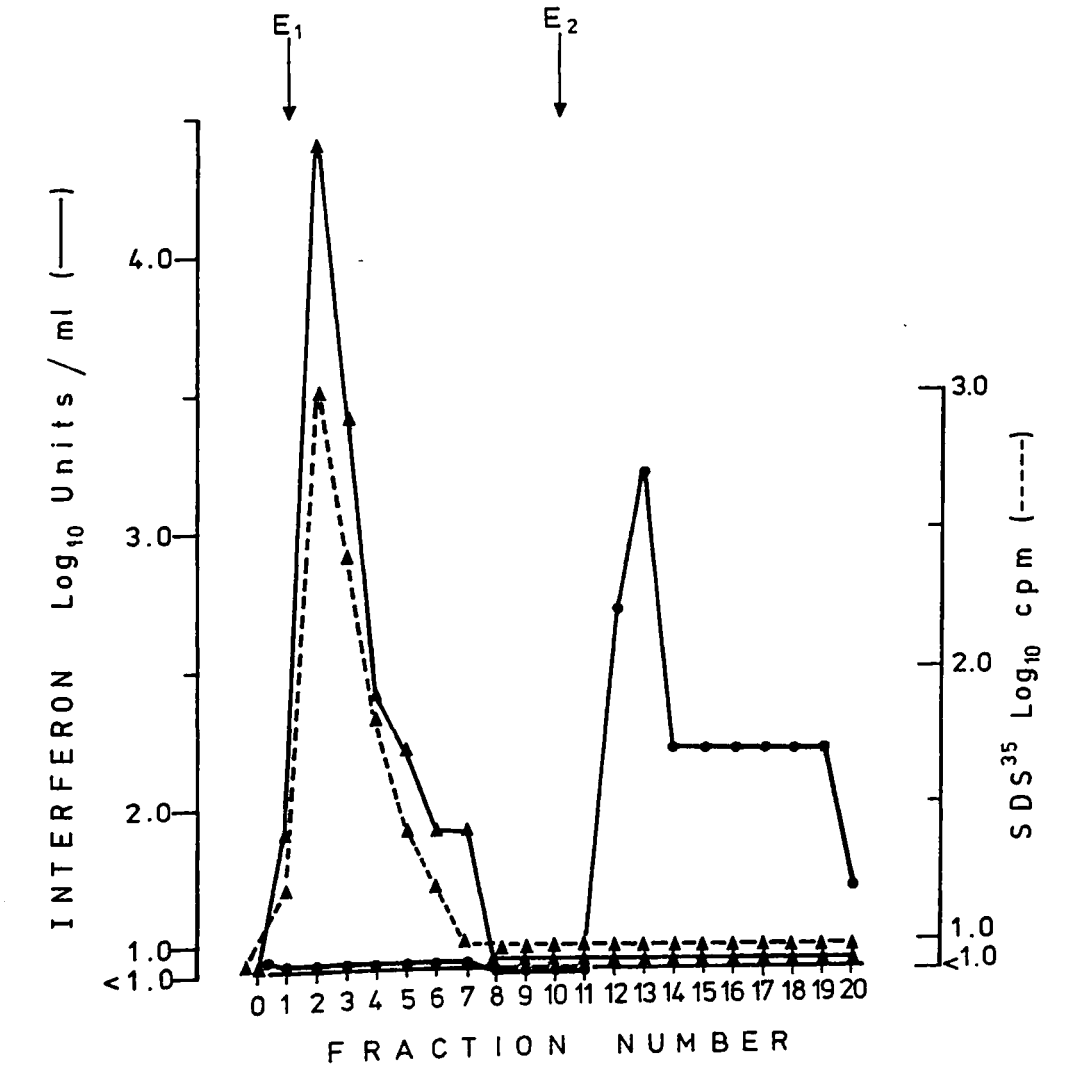

FIGURE 6. Chromatography of CPG/MIF, and SDS^{35}-CPG/MIF, on CPG. One ml samples of CPG/MIF (circles) and SDS^{35}-CPG/MIF (triangles), were loaded onto CPG. Collected fractions were assayed for interferon (solid lines) and SDS^{35} (broken line). The eluant PBS is denoted as E_1, and 400 mM gly-HCl, pH 2.0, as E_2.

rial, the amount of protein-associated SDS remained the same, and thus the change in the elution characteristics cannot be attributed to excess SDS.

Rechromatographed CPG/MIF was detected only in the acid wash. However, from the initial 5.0×10^4 units loaded, only 6.4 %, or 3.2×10^3 units of activity, were recovered. The alkali wash contained no detectable activity.

Thus, preparations, characterized as CPG-binding material, and of a higher specific activity, when treated with SDS, did not adsorb to CPG when rechromatographed.

SDS^{35}-AmS/MIF, passed through a column of AG1-X10 prior to loading onto CPG, exhibited an unusual elution profile (Figure 7). Of the 2.8×10^4 units of activity, and 1.8×10^3 cpm of SDS^{35}, the drop-through fractions contained only 5 % (1.3×10^3 units) and 33 % (6.0×10^2 cpm), respectively. Fractions obtained from the acid-wash had only residual counts but contained 15 % (4.2×10^3 units) of the starting material. The remaining 72 % (1.3×10^3 cpm) SDS^{35} was recovered in the subsequent alkali wash, but only 8 % (2.2×10^3 units) of the interferon. Therefore, despite the low interferon recoveries, all of the loaded SDS^{35} was collected. In addition, some of the detergent-treated material eluted in the acid wash, without containing detectable amounts of SDS^{35}.

Hydrophobic Chromatography of MIF, and SDS-MIF on Hexyl-Agarose

Since the dominant chemical property of SDS is its hydrophobicity, it would be useful to study the binding characteristics of SDS on hydrophobic columns and apply that knowledge in determining whether the tails of SDS, associated with interferons, are accessible to insolubilized hydrocarbons.

Figure 8 shows the binding characteristics of SDS^{35} on alkyl columns containing 0, 2, 4, 6, 8, or 10 carbon residues. One tenth of a ml SDS^{35}, diluted to contain 2.1×10^3 cpm, was loaded onto 1 ml columns of alkyl agarose, washed with 2 ml Tris-HCl, and the eluants determined for SDS^{35} content.

SDS^{35}-binding was noted only with hexyl-, octyl- and decyl-agarose, where no more than 12 to 15 % of the starting material eluted. When columns were subsequently washed with Tris-HCl containing 10 M ethylene glycol +

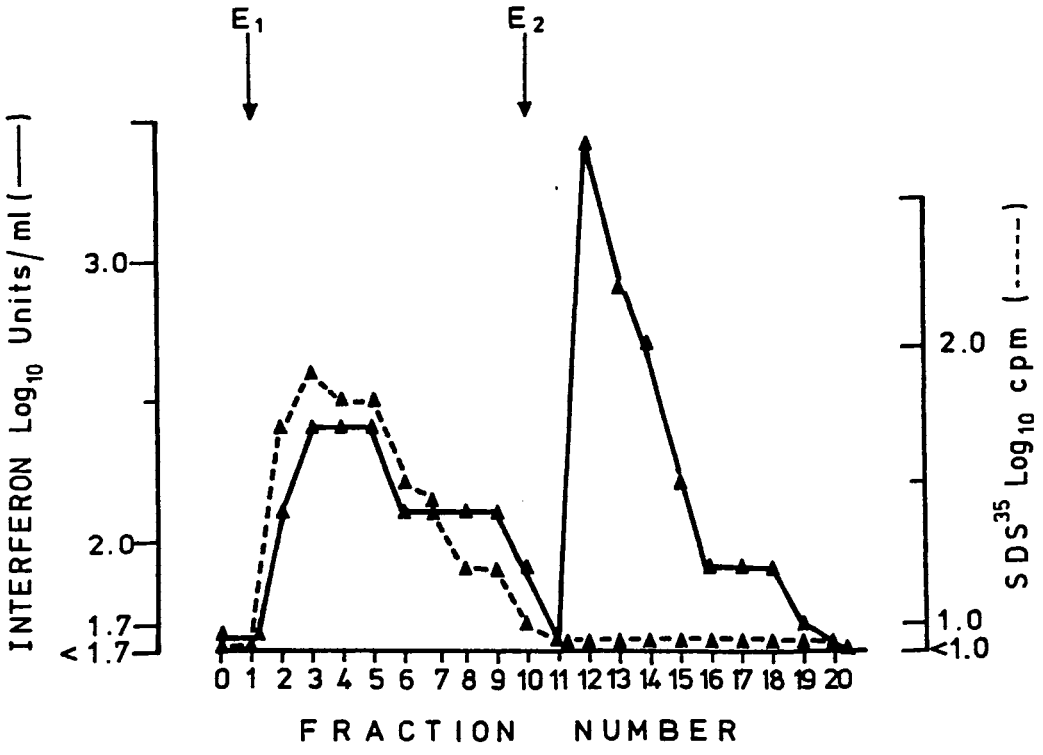

FIGURE 7. Chromatography of SDS^{35}-AmS/MIF passed through AG1-X10 prior to loading onto CPG. A 3 ml sample of SDS^{35}-AmS/MIF, passed through a column of AG1-X10, was loaded onto CPG. Collected fractions were assayed for interferon (solid line) and SDS^{35} (broken line). The eluant PBS is denoted as E_1, and 400 mM gly-HCl, pH 2.0, as E_2.

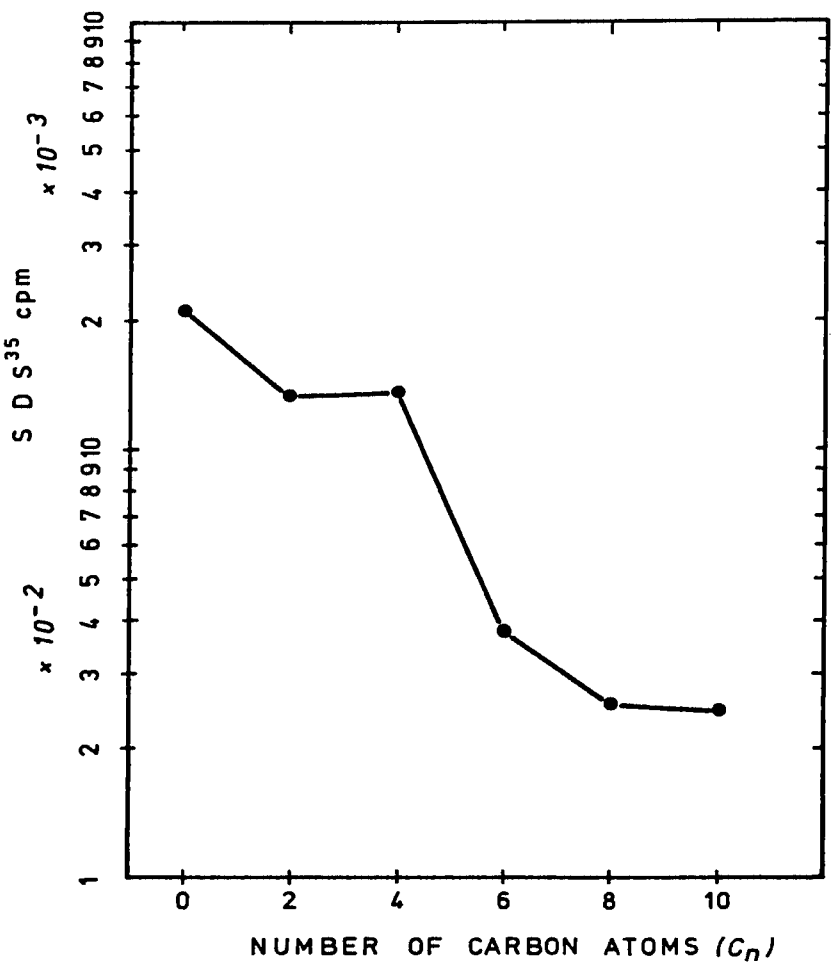

FIGURE 8. SDS^{35} binding versus number of carbon atoms per alkyl chain. One-tenth ml of SDS^{35} was loaded onto 1 ml columns of agarose only (Cn = 0), ethyl- (Cn = 2), butyl- (Cn = 4), hexyl- (Cn = 6), octyl- (Cn = 8), and decyl (Cn = 10) agarose, equilibrated in Tris-HCl buffer. Columns were washed with 2 ml Tris-HCl, and the eluants assayed for SDS^{35}.

1 M NaCl (EG-NaCl), more than 100 % (2.2 x 10^3 cpm) of the SDS^{35} was recovered from hexyl-agarose, while, only 0.04 % (9.7 x 10^1 cpm) was recovered from octyl-agarose. Thus, by virtue of its hydrophobicity, SDS^{35} can selectively, and in the case of octyl-agarose, essentially irreversibly, be bound to columns of hexyl-, octyl- and decyl-agarose.

Davey et al. (32), have previously reported that mouse interferon was unable to bind to hexyl-agarose, although it bound successfully to octyl-, and decyl-agarose. Thus, since the bulk of the SDS^{35} binds to hexyl-agarose, while MIF does not, examining the elution profile of SDS^{35}-CPG/MIF on hexyl-agarose would afford an excellent opportunity to further define the exact nature of the interaction of SDS with interferon. Because of the limited binding capacity of hexyl-agarose, CPG/MIF and SDS^{35}-MIF were employed in all experiments.

As indicated in Figure 9, SDS^{35}-CPG/MIF binds to hexyl-agarose, while greater than 90 % of the CPG/MIF does not. The drop-through fractions from CPG/MIF (1.6 x 10^5 units) contained 3.1 x 10^5 units of activity, or 194 % of the starting material. The samples obtained from the EG-NaCl wash represented only 14 % (2.2 x 10^4 units) of the starting material. But the drop-through fractions from SDS^{35}-CPG/MIF (3.2 x 10^4 units of interferon, and 2.8 x 10^3 cpm of SDS^{35}), had no detectable amounts of interferon, and only 11 % (3.2 x 10^2 cpm) of the SDS^{35}. Material obtained from the EG-NaCl wash contained only 1.6 % (5.0 x 10^2 units) and 36 % (1.0 x 10^3 cpm) of the interferon and SDS^{35}, respectively.

These results suggest that the hydrophobic tail of SDS, presumably associated with interferon, is accessible to hydrophobic binding with the immobilized hydrocarbon hexyl-agarose. The low antiviral recoveries from the EG-NaCl wash of SDS^{35}-CPG/MIF may be similar to what was observed with SDS^{35} bound to octyl-agarose (Figure 8). Perhaps MIF and SDS combined offers a similar effect.

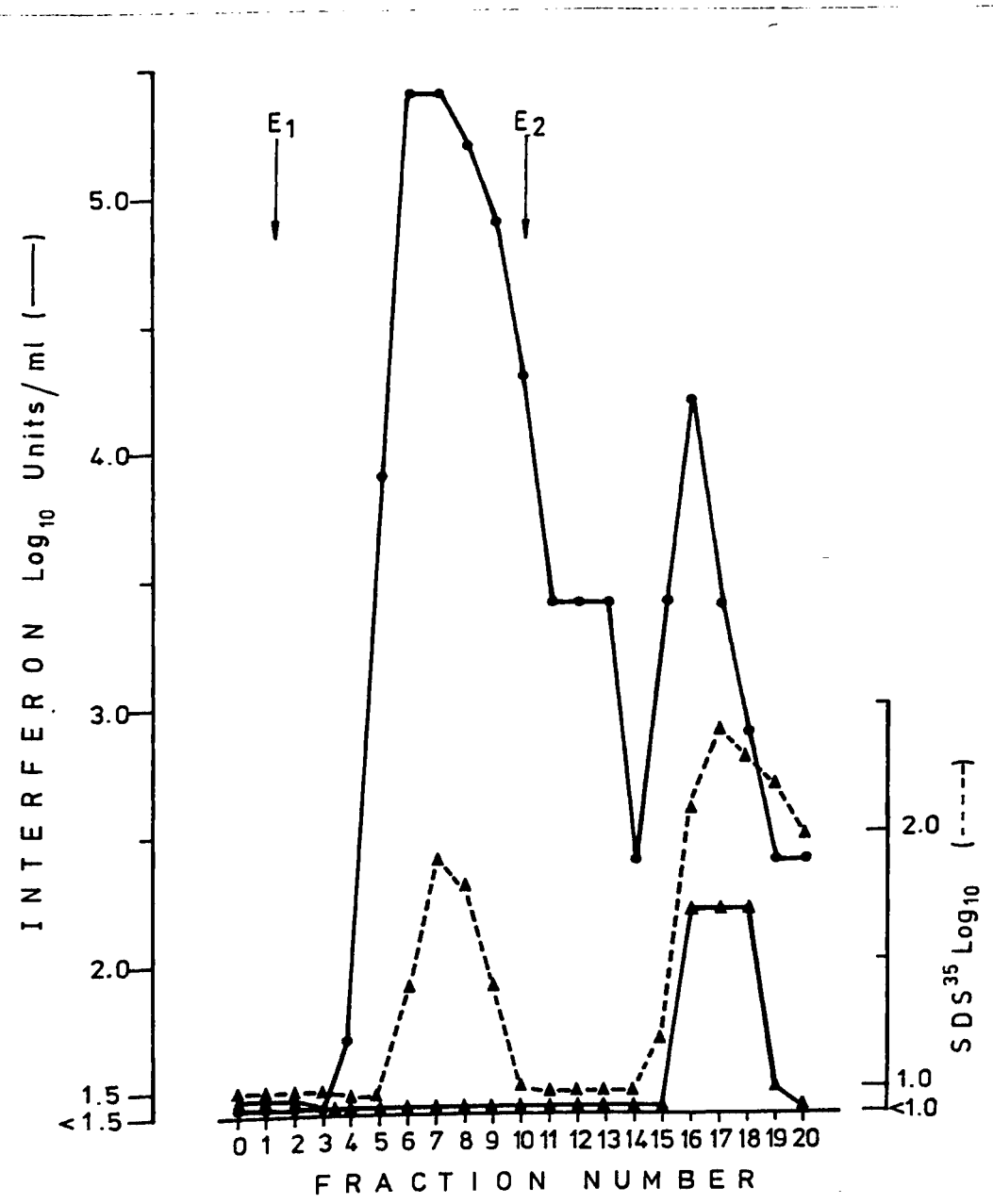

FIGURE 9. Chromatography of CPG/MIF and SDS^{35}-CPG/MIF on hexyl-agarose. One ml samples of CPG/MIF (circles) and SDS^{35}-CPG/MIF (triangles) were loaded onto hexyl-agarose. Collected fractions were assayed for interferon (solid lines) and SDS^{35} (broken line). The eluant 10 mM Tris-HCl, pH 7.6, containing 50 mM NaCl, is denoted as E_1, and PBS, containing 10 M ethylene glycol and 1 M NaCl as E_2.

Neutralization of MIF Treated with Sodium Dodecyl-, Tetradecyl-, and Hexadecyl Sulfate

The evidence thus far presented suggests that SDS, in some manner, is intimately associated with mouse interferon. Therefore, it would be interesting to examine whether SDS treated and untreated MIF respond differently to neutralization by specific anti-MIF-antibodies.

As indicated in Table 3, the antibody-neutralization endpoints of untreated AmS/MIF and AmS/MIF which had been treated with sodium dodecyl-, tetradecyl-, or hexadecyl sulfate were essentially the same. If the presence of these detergents did alter or contort the normal configuration of interferon, it was not reflected in the protein's antigenicity.

A Comparison of MIF and SDS-MIF Stability to Heat

Next, we examined the susceptibility of both SDS-MIF and MIF to exposure to 60°C, for 6 hr at pH 2.0 and 7.0. As indicated in Table 4, no differences were noted in the abilities of SDS-treated and untreated interferon to withstand heat. At pH 2.0, both SDS-AmS/MIF and MIF gained a slight (3-fold) increase in activity when exposed to 60°C for 6 hr. When heated at pH 7.0, both interferon preparations lost a significant part (about 90 %) of their activity (Table 4).

Although the starting materials had the same titer (the initial titers of AmS/MIF and SDS-AmS/MIF were not equivalent), slight variations occurred when, prior to heat treatment, samples were dialyzed at 23°C, for 18 hr, in their respective buffers.

The ability of mouse interferon to retain its activity when exposed to heat at low pH has been reported previously (60). Thus allowing for a 3-fold tolerance in the assays accuracy, the slight increase of activity observed with both SDS-MIF and MIF, after heat treatment, might be anticipated.

TABLE 3. Neutralization of MIF Treated with Sodium Dodecyl-, Tetradecyl-, and Hexadecyl Sulfate

AmS/MIF treated with[a] :	Neutralization Titer (log_{10} units/ml)
sodium dodecyl sulfate	4.2
sodium tetradecyl sulfate	3.9
sodium hexadecyl sulfate	4.2
none	4.2

(a) MIF treatment, with 3.5 mM detergent, was carried out at 100°C, for 10 min, in the presence of 2-ME and urea.

TABLE 4. Stability to Heat of SDS-MIF versus MIF

Description[a]	Before treatment (log_{10} units/ml)	After treatment (log_{10} units/ml)
SDS-MIF, pH 2.0	3.2	3.7
MIF, pH 2.0	2.2	2.7
SDS-MIF, pH 7.0	3.2	2.2
MIF, pH 7.0	2.7	1.7

(a) SDS-AmS/MIF and AmS/MIF, at pH 2.0 and 7.0, were treated with heat (60°C) for 6 hr. Samples were assayed before and after heat-treatment.

DISCUSSION

The goal of prior investigations, concerning treatment of mouse interferon with SDS, has been to establish the parameters necessary to stabilize and/or renature mouse interferon when subjected to a variety of denaturing conditions. However, the mechanism of stabilization, and the nature of the interaction between SDS and interferon, have not been elucidated. This report, then, was an attempt of studying this interaction, from three perspectives : (i), to define the structural requirements of the detergent necessary to stabilize mouse interferon in the presence of heat, urea, and 2-ME. Although it has been reported that supplementing SDS with these denaturants was required to promote full mouse interferon protection (122), in the absence of detergent, the denaturants rendered interferon inactive (122). Thus, SDS was tested for its ability to stabilize interferon against these conditions; (ii), employing anion exchange, CPG-adsorption, and hydrophobic chromatography, to determine the physical and chemical interactions between MIF and SDS; and (iii), compare the biological activities of detergent-treated and untreated MIF preparations' susceptibilities to heat and antibody.

The stabilizing effects of SDS on mouse interferon, in the presence of denaturants, can occur by several mechanisms : (a) the detergent can unfold the protein into a protective, reversible configuration, and upon its subsequent removal, the protein renatures; or (b), as in the case of serum albumin (80), the SDS prevents the protein from unwinding by forming detergent bridges between groups of nonpolar residues and charged amino groups located on different loops of the polypeptide chain; (c) alternatively, the protein does unfold, but the detergent bridges augment the renaturing process.

If, with interferon, the unfolding process is proportional to the extent of detergent hydrophobicity, as shown for other proteins (72), then, when tested in the absence of heat, urea, and 2-ME, the shorter alkyl sulfates should have left mouse interferon more active than the longer species. Since all of these compounds tested inactivated interferon to the same extent, the degree of protein unwinding conveyed by these alkyl sulfates appears to be similar. Furthermore, as shown in Figure 1, as boiling of these compounds in the presence of urea and 2-ME did not produce a gradual rise

in residual activities as a function of increasing molecular length, it would seem that the unfolding process is not playing a major part in stabilization.

The sharp increase in residual activities observed when going from decyl to dodecyl sulfate might be attributed to the additional 3 Å length necessary to extend between the appropriate nonpolar and positively-charged regions of the protein to form detergent bridges. These bridges could either prevent the molecule from denaturing, or augment the renaturing process by increasing the probability of the molecule reassuming an active conformation.

The latter assumption is the most likely for two reasons. First, despite the presence of SDS, proteins treated with urea, 2-ME, and boiled, will unfold (53). Second, evidence previously reported by Stewart et al. (122), and also obtained here, indicates that detergent-treated mouse interferon is not any more stable than untreated. Thus, SDS, bound to interferon, did not prevent the protein, when exposed to heat, from denaturation.

All of the water-soluble amphiphiles tested contained both a hydrophobic and an ionic moiety. However, except for 1-tetradecane sulfonate (which was only partially active with respect to SDS), none significantly stabilized mouse interferon against the presence of heat, urea and 2-ME. The comparatively low CMC of most of these compounds probably would not allow a sufficient concentration of monomer to become protein-bound. Furthermore, those which do bind, may not fulfill the physicochemical requirements necessary to stabilize mouse interferon.

It is interesting that although SDS and sodium tetradecyl sulfate stabilize MIF, in the presence of heat, urea and 2-ME, to the same extent (Figure 1), sodium 1-tetradecane sulfonate, which was less active than SDS (Table 1), was ten-times more active than sodium 1-dodecane sulfonate (Table 1). As the length of the molecule is critical for stabilizing MIF (Figure 1), perhaps, since with the sulfonates an oxygen atom is missing between the last carbon residue and sulfur, the length is compensated for, with sodium 1-tetradecane sulfonate, by the additional two carbon atoms, whereas, sodium 1-dodecane sulfonate is less active than SDS (Table 1) because of its shorter (about 1.4 Å) length. An alternative explanation would be the differences in the sulfate and sulfonate groups negatively charged environments.

The necessity of a negatively charged polar head within the detergent

to stabilize interferon is evidenced by the poor results achieved with dodecylamine. Although it meets the structural requirement for hydrocarbon length, unlike SDS, the polar portion is positively-charged.

Most surprisingly, lithium dodecyl sulfate, the compound most similar to SDS, was also inactive. Except for differences in the salts counter-ions, the two should be identical. However, since the type of counter-ion present can drastically effect the detergents CMC (53), perhaps the substitution of sodium for lithium reduced the monomeric species concentration necessary for effective protein-binding.

Since treatment with citraconic anhydride also reversibly stabilizes interferon, the amount of further protection conveyed by SDS cannot be discerned. However, as detergent-treated CT-MIF was ten-times more active than untreated CT-MIF (Table 2), and SDS partially reactivated CT-MIF prior to deblocking, it is tempting to speculate about the mechanism by which SDS restored activity, perhaps from an entirely different conformation directed by the presence of citraconic anhydride.

Since, the MIF preparations employed were not pure, the results obtained, from the column chromatography experiments, also apply for many, unidentified, proteins. Furthermore, because of the MIF preparation's impurity, SDS (measured by radioactivity) effects or the elution profile of MIF (detected by antiviral activity), and the converse, were the only means of identifying the association between SDS and MIF. Consequently, as, in some measure MIF in the presence of SDS is inactivated, and at this writing biological activity is the only means of identification, the fate of the chromatographed inactive material could not be determined.

Both SDS and MIF co-eluted from AG1-X10 column loaded with SDS-MIF (Figure 2). As this column has a large binding capacity for free SDS, and does not bind MIF, the detergent, in some manner, must have been associated with the protein. Despite the restricted pore size of the bead, the possibility remains that the resins quaternary ammonium groups may have stripped off protein-bound SDS, or immobilized a minor part of detergent-protein complexes.

Unlike the results obtained with AG1-X10, when detergent-treated and untreated MIF preparations were chromatographed on AG1-X2, the former bound to the column while the latter did not (Figure 3). Due to its low-percentage cross-linking, the capacity of this resin is not as great as AG1-X10.

However, a 1 ml column can bind at least 100 mg SDS (135), which is ample to accomodate the amount of SDS-MIF applied. As the amphiphile's polar sulfate group contains three negative charges, if only one interacts with the necessary basic amino acid, the remaining two are conceivably available for binding with the quaternary ammonium groups on the matrix, thus immobilizing the detergent-protein complex. This would suggest, at least, that the detergent's head is located on the surface of the protein.

The CPG-adsorption characteristics of MIF were fundamentally altered upon SDS treatment. This was particularly shown with CPG-purified interferon preparations which were detergent-treated and rechromatographed on CPG (Figure 6).

Although an hydrophobic interaction cannot be disregarded, the binding between CPG, and acid-elutable proteins, such as MIF, are predominantly silanol-basic amino acid interactions (Chapter 3). Hydrophobic eluants, such as ethylene glycol and high salt concentrations, in as much as they desorb a proportion of all bound proteins, including acid- and alkali-elutable ininterferons, are more non-specific (Chapter 3). Therefore, since some of the SDS-MIF eluted only with an alkali buffer, it may be concluded that a slight hydrophobic interaction occurs, and that SDS does not mask this site.

The starting material (SDS-MIF), after dialysis, contained insufficient SDS to contribute to a non-specific elution of interferon. Therefore the presence of SDS must have either specifically altered or masked the proteins, presumably basic amino acid regions, CPG-binding sites.

The reversion to a normal CPG elution profile with SDS-MIF chromatographed on AG1-X10 prior to loading onto CPG cannot be easily explained. One speculation, however, is the following : since the CMC of SDS limits the monomer concentration, perhaps the saturation of high affinity binding sites for all of the sample proteins was not achieved. Consequently, due to differences in cooperative binding, not all proteins unfoled to the same extent, and thus were not in a position to trap SDS within their subsurface during the renaturing process. Upon dialysis, the product then would contain a heterogenous population of detergent-containing proteins. Conceivably, some proteins contain both surface and subsurface SDS (140), while others contained the former only. When these preparations were passed through AG1-X10, some surface-bound SDS was removed, the interferon remained active, and behaved as untreated MIF. That, both in the drop-through and the

alkali-wash interferon and SDS co-eluted, may represent proteins containing SDS trapped within its surface.

Another contrast between detergent-treated and untreated preparations was observed with hexyl-agarose hydrophobic chromatography. SDS-MIF and essentially all of the free SDS were bound to the immobilized hydrocarbon, while as previously reported (32), the majority of MIF was not. This implies that, in addition to the polar region, the amphiphile's nonpolar tail can also reside, presumably by hydrophobic interactions, on the surface of the protein. The existence of hydrophobic patches, located on the protein's surface and accessible to SDS binding, has been reported to occur for albumin (53), and may exist for beta-lactoglobulin (137). It has also been hypothesized that human interferons may also possess such surface regions (129).

It was also interesting that, following washing of the columns in EG-NaCl, half of the input SDS eluted, but only a small portion of the interferon. This might have been possible if, once exposed to the eluant, some of the surface-bound detergent was stripped from the protein, while, possibly due to ionic interactions or physical restrictions, the more tightly-bound SDS continues to immobilize interferon. Alternatively, while under desorbing conditions, the removal of the SDS may have altered the conformation of interferon (either into an active or inactive form), so that its sequestered hydrophobic regions became available for binding to hexyl-agarose.

The small portion of untreated MIF which did bind to hexyl-agarose may represent a minor interferon subpopulation. Similar interferon subpopulations with physicochemical characteristics, that are different from the main interferon population, have been described (37,67,118,123,127). Since bound MIF eluted with EG-NaCl, while SDS-MIF did not, it can be excluded that the detergent-treatment converted MIF to the aberrant interferon subpopulation.

As far as neutralization by antibody and resistance to heat are concerned, no significant differences were detected in the biological behavior of SDS-MIF and MIF. If the conformation of mouse interferon became distorted when bound to either dodecyl-, tetradecyl-, or hexadecyl sulfate, it was not sufficient to alter the protein's antigenicity. This phenomenon has previously been observed for other proteins (29,104,128), including

human leukocyte interferon (9). However, variations in the antigenicity of MIF treated with these detergents may have been detected with heterologous antisera (i.e., anti-human leukocyte interferon).

The evidence reported here suggests that although SDS unfolds mouse interferon, it subsequently stabilizes the interferon molecule during the renaturing process, possibly via detergent bridges which promote the proper refolding pathway. Furthermore, even after dialysis in either serum-containing medium or PBS, SDS remains bound to the protein.

Whether detergent-treated interferon is structurally identical to untreated could not be determined. Crystallographic studies have shown that SDS bound to triclinic lysozyme did alter the protein's conformation (140). This observation has also been described for human leukocyte interferon (3). If true, this might explain why the lower molecular weight component of detergent-associated human leukocyte interferon, treated in the presence of urea and 2-ME, lost its homologous, but not its heterologous activity on cat cells (39). Urea, 2-ME and heat, all increase the extent of protein unfolding so that SDS can bind to hidden regions of the protein (53). Possibly then, upon refolding, the trapped SDS destroy human leukocyte interferon's active conformation for the homologous receptor, but not the heterologous. This hypothesis might also explain the different response to SDS-treatment, in the presence and absence of reducing agents, of mouse interferon (118,123,127) and human leukocyte interferon subpopulations (125). An interesting sequel of experiment, then, would be to examine the elution profiles, of these populations, in the various chromatographic systems employed in this report.

SDS uniqueness as a stabilizing agent was ultimately due to its physicochemical characteristics. This was manifested not only in its relatively high CMC, which permitted a large monomeric concentration to interact with proteins, but also in its ability to form intramolecular bridges.

The limiting factor in these detergent-protein interactions was the availability of monomeric SDS. If the specific SDS-interferon ratios required for stability were not reached for the entire population, it would explain why some detergent-mediated inactivation occurred. Since the CMC of SDS is 8.2 mM (53), this explanation can only be resolved in two ways : dilute the protein concentration of the starting material, which would not increase the specific SDS-interferon ratios; or, redefine the parameters

and employ interferon preparations of a higher purity.

It would also be interesting to treat interferons, derived from other species, with sodium alkyl sulfates of various lengths, and determine whether the same structural features apply for those interferons as established here for mouse interferon.

SUMMARY

1. The capacity of various alkyl sulfates to stabilize mouse interferon (MIF) (in the presence of urea, 2-mercaptoethanol, and heat), is dependent upon the molecular length of the alkyl residue (critical length of the molecule $\geq$ 22 Å or 12 carbon atoms). Furthermore, since all alkyl sulfates (in the absence of urea, 2-ME, and heat) inactivate MIF to the same extent, the stabilizing effects of sodium dodecyl sulfate (SDS) most likely occur during the refolding process due to the formation of intramolecular detergent bridges.
2. Unlike SDS, other amphiphiles, such as dodecylamine, lithium dodecyl sulfate, and sodium 1-dodecane sulfonate, did not have an appreciable stabilizing effect on MIF.
3. After it has reacted with interferon, a portion (about 20 - 50 %) of the SDS is no longer retained by the anion exchange resin AG1-X10. Conversely, interferon (which does not bind to AG1-X2, a resin with a larger pore size than AG1-X10) is retained by the resin AG1-X2 upon reaction with SDS.
4. After it has reacted with SDS, MIF no longer binds to controlled pore glass (CPG). This is most likely due to SDS masking of the proteins' silanol-binding sites, whether they be basic amino acid residues or hydrophobic.
5. That interferon-associated SDS is able to bind to hexyl-agarose (under conditions where the majority of the MIF, itself, is not retained) suggests that some proportion of the SDS adheres to the surface of MIF.
6. There is no difference between the susceptibilities of SDS-treated and untreated MIF to either heat or neutralizing antibodies.
7. In as much as some SDS co-elutes with MIF from AG1-X10, it is concluded that SDS does bind to MIF. Furthermore, since SDS-treated MIF binds to AG1-X2 and hexyl-agarose, then at least some of the detergents polar and hydrophobic regions are located on the surface of the protein.

CHAPTER THREE

CONCERT CHROMATOGRAPHY OF MOUSE INTERFERON

INTRODUCTION

Until recently, the purification of interferons has ranked amongst one of the most frustrating pursuits in the field. The lack of significant progress can be attributed to three features of these glycoproteins. First, interferons are molecules with an extremely high biologic activity. With a predicted specific activity of greater than 1.0×10^9 units/mg protein (92), one unit of activity would constitute less than one picogram protein. Second, inactivation readily occurs when the protein concentration is reduced to less than 10 - 25 μg/ml (67,69). Lastly, interferons' nonspecifc "stickyness" to the apparatus employed, usually yields low recoveries (119). Therefore, a successful purification would require an enormous amount of starting material. Indeed, with the advent of mass interferon production and the judicious use of interferons' unique properties, products with a specific activity of greater than 1.0×10^9 units/mg have been achieved (37,62).

Following recent trends in protein purification, interferon has been separated from its neighbor molecules by : selecting for differences in physicochemical characteristics, the use of anti-interferon or anti-impurity antibodies, and some very novel affinity ligands and/or adsorbents. Employing the more classical, and perhaps archaic, techniques, interferons have been purified by size, with gel filtration chromatography (38,61,62,67,69, 81,131); charge, with either ionic exchange chromatography or isoelectric focusing (18,38,61,69,81); solubility in acid or salt (18,61,62,67,69); and electrophoretic mobility, with polyacrylamide gel electrophoresis (PAGE) (61). Interestingly, despite the many reports concerning SDS' ability to stabilize interferons (121,122,123,125), SDS-PAGE has been employed only as an analytic tool (37,62,69,118), and not as a method of purification.

Usually only a ten-fold purification was obtained by any of the above techniques if applied individually. However, when ammonium sulfate precipitation, gel filtration, and ionic exchange chromatography were employed in a sequential manner, a specific activity of greater than 1.0×10^8 units/mg was achieved for both mouse L-929 interferon (61,67), and human diploid fibroblast interferon (69).

Antibodies, raised in an assortment of animals, have been immobilized on a solid support and employed as a successful tool for purifying interferons (112). If, prior to insolubilization, antibodies specific for the

"contaminants", are selectively removed, this application would then be an ideal one-step procedure, as it offers the advantage of having both a large, and specific, loading capacity.

Ogburn et al. (94), pioneered this latter approach, achieving a specific activity of greater than 1.0×10^8 units/mg for mouse L-929 interferon (MIF). Later, Berg (9), refined the technique, and obtained approximately the same purity for human leukocyte interferon. The concept of a "scavenger" column was also reported by Anfinsen et al. (4). Further improvements, applied to mouse C-243 interferon, led to a product with a specific activity of greater than 1.0×10^9 units/mg (37).

An alternative to removing antibodies directed against contaminants would be first to purify interferons, by some means, to the highest specific activity obtainable, and employ that product as the immunogen. In fact, this option has proven quite successful and the purified material extremely immunogenic (Peter Lengyel, personal communication).

In recent years, the impetus in interferon purification has been towards affinity- and/or adsorption chromatography. Similiar to immunoabsorbents, columns of immobilized ligands usually possess a high specificity and capacity, and thus achieve a high degree of purification. Utilizing one such ligands, as much as a three-thousand-fold purification, in a single step, has been reported (42).

By virtue of their hybrophobicity, and possibly their charge, interferons have been separated from their neighbors with columns containing such diverse immobilized ligands as albumin (33,56), hydrocarbons, oligopeptides and amino acids (32,129), blue dextran (16,35), and AFFI-Gel 202 and CH-Sepharose (20,33). Neither the specificity nor the precise interaction involved are clearly understood. Depending upon the species of origin, inteferons vary to their ligand affinities (16) and it has been hypothesized that interferons contain specific hydrophobic pockets (129), located either on its surface or subsurface. Human fibroblast interferons have also been purified by Concanavalin A-Agarose, which required both carbohydrate and hydrophobic interferons (31).

De Maeyer and colleagues hypothesized that since mouse interferons bound to blue dextran (35), they might also contain a dinucleotide fold (130). They therefore extended their investigations to include various polynucleotides as possible ligands for interferon purification. Mouse inter-

ferons did bind to a variety of polynucleotides, most notably poly(U) where a hundred-fold purification, to a specific activity in excess of 1.0×10^9 units/mg, was achieved (36).

Metal chelate affinity chromatography (102), has also been applied to hamster (15), and human fibroblast (42) interferons. The latter, employing zinc, achieved a specific activity, in one-step, of 3.0×10^8 units/mg. Human fibroblast interferon has also been reported to have been purified on columns containing neuraminidase (46) and Thiol-Sepharose (22).

Recently, two groups, applying a sequential approach, have reported a specific activity for mouse interferon of greater than 1.0×10^9 units/mg. De Maeyer-Guignard et al. (37), purified mouse C-243 interferon first on Poly(U)-Sepharose followed by immunoabsorbent, chromatography. The other, Kawakita et al. (62), purified mouse Ehrlich ascites cell interferon by combining ammonium sulfate precipitation, CM-Sephadex, blue-dextran, gel filtration with Bio-Gel P-60 and P-200, phosphocellulose, isoelectric focusing, and hydrophobic chromatography with Octyl-Sepharose.

A comparison of the two procedures employed offers proof of the increased efficiency of the immunoabsorbents and affinity ligands over the more prodominately classical approach. Despite differences in the specific activity of the starting material, the former method required only 2.6×10^8 units of starting material and achieved a recovery of 52 %. The latter, required 2.3×10^9 units of starting material, but only a 2 % recovery was obtained.

Organomercurial affinity chromatography has also been applied to a variety of proteins (13,90,109,115). This ligand is a useful probe for identifying cysteine-containing proteins, and as reported here, mouse inteferon belongs to this class of proteins.

Controlled pore glass (CPG), was originally utilized as a molecular sieve (27,50). However, some proteins (11,86) bound to the beads, and when alkali and/or chaotrophic buffers were employed, their selective elution was obtained. Mouse L-929 and human fibroblast interferons also adsorb to CPG, but unlike the above proteins, elute under acidic conditions (43). It is this distinction which permits the high degree of purification of some interferons on CPG.

This chapter describes, in greater detail, the purification of mouse interferon on CPG. In addition, evidence is provided suggesting that the

binding of mouse interferon to CPG occurs via regions of high-lysine and arginine content, and the hydroxyl groups of the beads. Hydrophobic binding, but of a less specific nature, may also be involved.

In addition, two schemes are described to purify mouse interferon which are based on the concerted application of various physicochemical and affinity/adsorption column chromatographic techniques. Both, by coordinating the eluting and loading buffers employed, were designed to limit the amount of time and manipulations (e.g. dialysis and concentration) and thereby reduce some of the inherent problems in purifying interferons.

The first scheme, which yielded a 59 % recovery, and where the peak material had a specific activity of 8.0×10^8 units/mg, or a 116-fold purification, consisted of hydrophobic chromatography, with AFFI-Gel 202, and CPG-adsorption chromatography. The second, which yielded only 6.7 % recovery, and where the specific activity of the peak material was 3.7×10^8 units/mg protein, or 41-fold purification, employed, prior to the two steps described above, gel filtration, with Ultrogel AcA 54, and ionic exchange chromatography, with Carboxymethyl Bio-Gel Agarose.

MATERIALS AND METHODS

Chemicals

Capsid proteins from TMV (purified virus) was a generous gift from Prof. Jeener (Molecular Biology Dept. ULB, Belgium), cytochrome c, horse myoglobin, ovalbumin, bovine serum albumin, and DNP-l-α-alanine were obtained from Serva Feinbiochemical (Heidelberg, W.Germany), AFFI-Gel 202 and Carboxymethyl Bio-Gel Agarose (CM-BGA) from Bio-Rad (Richmond, Calif.), Ultrogel AcA 54 from LKB (Bromma, Sweden), Agarose-p-aminophenyl mercuric acetate from Miles Laboratories (Elkhart, Ind.), Controlled Pore Glass (CPG) from Electro-Nucleonics (Fairfield, N.J.), human plasma protein fraction (HPP) from the National Blood Transfusion Service (Belgian Red Cross), poly-l-lysine (15,000 - 30,000 MW), poly-l-arginine (15,000 - 50,000 MW) and insulin from Sigma Chemical Co. (St. Louis, Mo.), zirconium tetrachloride ($ZrCl_4$) from Merck (Darmstadt, W.Germany), aprotinin (Trasylol) and asparaginase (Crasnitine) from Bayer (Leverkusen, W.Germany), and lysozyme (crystallized from egg white) and bovine albumin (fraction V) from Armour Pharmaceutical (Eastbourne, England).

Ammonium Sulfate Precipitation

The procedure was essentially the same as described by Knight (67). The pH of crude mouse L-929 interferon was adjusted to 2.0 with 12 M HCl. Solid ammonium sulfate, $(NH_4)_2SO_4$, was added to make a 40 % saturated salt solution, and while stirring, allowed to incubate at 23°C, for 15 min. The pH was maintained at 2.0 with 2 M HCl. The material was then spun at 1400 x g, at 4°C, for 30 min, and the salt saturation level of the supernatant raised to 65 % with solid $(NH_4)_2SO_4$. This mixture was incubated for an additional 15 min, at 23°C, recentrifuged, the supernatant decanted, and the pellet resuspended in 10 ml 50 mM sodium acetate (NaAc) buffer, pH 5.0. The salt was removed by dialyzing the material against the same buffer. The average increase in purification was approximately 10 - 50-fold, with specific activities ranging from 1.0 - 90.0 x 10^5 units/mg. This preparation is referred to as 1°-MIF throughout the text.

Gel Filtration Chromatography with Ultrogel AcA 54

The procedure was modeled after the technique described by Knight (67). A 1.6 x 63 cm column was packed with Ultrogel AcA 54 and equilibrated in 50 mM potassium dihydrogen phosphate buffer (K-PB), pH 5.9. 1°-MIF was dialyzed against the equilibration buffer and concentrated with 80 % saturation $(NH_4)_2SO_4$ as follows : solid $(NH_4)_2SO_4$ was added to 1°-MIF, and while stirring, allowed to incubate at 23°C, for 30 min. The mixture was then pelleted at 1400 x g, for 30 min, at 4°C, and the precipitant resuspended in 2 ml equilibration buffer. Five µl of DNP-l-α-alanine was added as a marker, and the sample loaded onto the column. The flow rate was 10 ml/hr, and 80-2 ml fractions were collected. The optial extinctions at 280 nm (OD_{280}) of the column effluent was measured with LKB Uvicord III ultraviolet absorption monitor (LKB Produkter, A.B., Bromma, Sweden), using a 3 mm optical path length continuous flow cell. In the concert chromatography experiments, interferon purified by this procedure, is referred to in the text as 2°-MIF, in scheme 2.

Ionic Exchange Chromatography with CM-BGA

This procedure is also a variation of the technique previously described by Knight (67). A 0.7 x 1.5 cm column of CM-BGA was equilibrated in 50 mM K-PB, pH 5.9, and the flow rate adjusted to 10 ml/hr. The peak fractions from 2°-MIF, scheme 2, were loaded directly onto CM-BGA, and the column washed in equilibration buffer. The bulk of the activity was then eluted with 100 mM K-PB, pH 8.0, and 1 ml fractions were collected. Absorption at 280 nm was detected as described above. In the concert chromatography experiments, interferon, purified by this procedure, is referred to in the text as 3°-MIF, in scheme 2.

Hydrophobic Chromatography with AFFI-Gel 202

This procedure has been previously described by Davey et al. (33). The column sizes employed varied according to their use, and are further

described in the text. Depending upon the experiment or scheme, columns, having been regenerated with PBS containing 10 M ethylene glycol and 1 M NaCl, were equilibrated either with 50 mM K-PB or NaAc, pH 5.0.

After loading the starting material, columns were washed with the appropriate equilibration buffer, followed by 20 mM sodium phosphate buffer (Na-PB), pH 7.2. The bulk of the activity was then eluted with PBS containing 350 mM NaCl (PBS + 350 mM NaCl). Absorption at 280 nm was detected as described above. In the concert chromatography experiments, interferon, purified by this procedure, is referred to in the text as either 2°-MIF or 4°-MIF, in schemes 1 and 2 respectively.

CPG-Adsorption Chromatography

CPG bends were regenerated by washing in 65 % nitric acid until the yellow colored suspension turned clear. This was followed by extensively washing the suspension in double distilled water until the pH reached 5.0. The beads were then equilibrated in PBS.

Depending upon their use, various size columns were employed which are further described in the text. In addition, a variety of elution procedures were tested, which are also deferred to the results section.

Absorption at 280 nm was detected as previously described. In the concert chromatography experiments, interferon, purified by this procedure, is referred to in the text as either 3°-MIF or 5°-MIF, in schemes 1 and 2 respectively.

Citraconylation of MIF

The citraconylation, and its subsequent deblocking, of 1°-MIF were described in Chapter 2.

CPG-Adsorption Chromatography of Poly-l-lysine and Poly-l-Arginine

One-hundred µg of poly-l-lysine or poly-l-arginine, in 1 ml PBS, was loaded onto a 1 x 3 cm column of CPG, equilibrated in the same buffer. The column was then washed with PBS, followed by 10 mM Tris-HCl buffer, pH 8.9, and 400 mM glycine-HCl (gly-HCl) buffer, pH 2.0. Ten-1 ml fractions of each were collected, and prior to spectrophotometrically (at 208 nm) determining the polymer content of each fraction, in a Beckman spectrophotometer (Model number 25), samples containing Tris-HCl and gly-HCl were dialyzed against PBS.

ZrOH-Adsorption Chromatography

Zirconium hydroxide (ZrOH) was prepared essentially as described by Kennedy et al. (63). While stirring, 1.5 g of $ZrCl_4$ was slowly added to 10 ml 1 M HCl. The pH was then gradually increased to 7.0 with 2.0 M NaOH. The slower the rate of increase in pH, the greater the size of polymer. The grains were then washed with 3 liters saline, followed by 1 liter PBS.

Crude MIF was purified on ZrOH in both a batch and column procedure. In the former, 100 ml of MIF was added to 10 ml packed ZrOH, and while stirring, incubated for 2 hr, at 23°C. The grains were then washed 5-times with 100 ml of PBS, and once with 100 ml of 400 mM gly-HCl, pH 3.5. Finally, the settled material was washed with 10 ml 400 mM gly-HCl, pH 2.0, and while stirring, incubated at 23°C, for 1 hr. During the procedure, 1 ml samples from each wash, were removed and assayed for antiviral activity.

In the latter technique, 10 ml MIF was loaded onto a 0.5 x 5.0 cm column of ZrOH, equilibrated in PBS. The column was then washed in the equilibration buffer, followed by 400 mM gly-HCl, pH 3.5, and, 4-4 ml and 1-4 ml fractions, respectively collected. Finally, the packed material was washed with 400 mM gly-HCl, pH 2.0, and 5-1 ml fractions collected.

Organomercurial Affinity Chromatography

In a 0.5 x 5.0 cm column, agarose-p-aminophenyl mercuric acetate (Hg-agarose) was regenerated in 50 mM NaAc, pH 5.0, containing 10 mM $HgCl_2$ and 20 mM EDTA. The excess $HgCl_2$ was then removed with 100 mM Na-PB, pH 6.2, containing 200 mM NaCl and 1 mM EDTA. The column was then equilibrated in 100 mM Na-PB, pH 8.0. One ml 1°-MIF, pre-dialyzed against the equilibration buffer, was loaded and the column washed in the same. The column was then washed with 50 mM NaAc, pH 5.0, followed by 50 mM NaAc, pH 5.0, containing 0.5 mM or 2.5 mM cysteine. The absorption at 280 nm was detected as described above.

Concert Chromatography

Scheme 1

Crude MIF was concentrated and purified by $(NH_4)_2SO_4$ precipitation (1°-MIF), and dialyzed against 50 mM NaAc, pH 5.0. This was then loaded (flow rate 10-18 ml/hr) directed onto a 0.9 x 12 cm column of AFFI-GEL 202, equilibrated in 50 mM NaAc, pH 5.0. After column washes with equilibration buffer (30-180 ml) and 20 mM Na-PB, pH 7.2 (60 ml), the material (2°-MIF) was eluted with 72 ml of PBS containing 350 mM NaCl, pH 7.2. During this last step, the output from the column was pumped directly onto the input portion of the CPG column (either 0.9 x 12 cm or 0.7 x 5.3 cm), equilibrated in PBS. Upon completion of this step, the first column was disconnected. The CPG column was then washed with 30-180 ml PBS, followed by 30-60 ml 400 mM gly-HCl, pH 3.5. Finally, the column was washed with 400 mM gly-HCl, pH 2.0, and 1 ml fractions collected. All fractions with a pH less than 3.5 were assayed for antiviral activity (3°-MIF).

Scheme 2

Crude MIF was concentrated and purified, as described above, and dialyzed against 50 mM K-PB, pH 5.9. This was then concentrated with 80 % saturation $(NH_4)_2SO_4$ and loaded (flow rate 10 ml/hr) onto a 1.6 x 63 cm column of Ultrogel AcA 54, as described above. The peak activity fractions (2°-MIF), were then directly loaded (flow rate 10 ml/hr) onto a 1.0 x 1.5 cm

column of CM-BGA, equilibrated in 50 mM K-PB, pH 5.9. As described above, the column was then washed in equilibration buffer (10 ml), and the activity (3°-MIF) eluted with 10 ml 100 mM K-PB, pH 8.0. The peak protein-containing samples were pooled, diluted 2-fold in double-distilled water, then pH-adjusted with 1 M HCl, to 5.0, and applied to a 0.7 x 5.3 cm column of AFFI-Gel 202, equilibrated in 50 mM K-PB, pH 5.0. After washing the column with equilibration buffer, and 20 mM Na-PB, pH 7.2, (10 ml each), as described in scheme 1, the format for the chromatography from AFFI-Gel (4°-MIF) to CPG (0.7 x 5.3 cm column) was employed. The subsequent CPG column washes and elution of activity (5°-MIF) were also described in scheme 1.

Protein Determinations

The protein content of samples was determined by the method described by either Lowry et al. (77), or Böhlen et al. (14).

SDS-PAGE

Interferon preparations or markers were added to the sample buffer so that the final concentrations were as follows : sucrose 10 %, SDS 1%, urea 1.7 %, 400 mM glycine, and 50 mM Tris-HCl, pH 6.8. Samples were then boiled for 1 min and loaded (130 µl for preparative gels and 20 µl for analytical) onto a vertical slab gel. The lower gel (containing 15 % acrylamide and 0.08 % BIS) was 13 cm in height and either 3 mm thick for preparative gels or 1.5 mm for analytical gels. The upper gel (containing 3 % acrylamide and 0.8 % BIS) was 3 cm in height and either 3 mm or 1.5 mm thick for preparative and analytical gels, respectively. The running buffer was as previously described by Laemmli (71).

Samples were electrophoresed at 50 V, at room temperature for 20 hr. The preparative gel was sliced into 0.5 cm sections and placed into glass tubes containing 0.5 ml running buffer and 0.4 % BSA. Sections were then assayed for eluted antiviral activity.

The markers in the preparative gel and all samples from the analytical gel were stained for protein as follows : gels were stained for 1 hr in 0.4 %. Coomassie brilliant blue containing 50 % methanol and 7 % acetic

acid, and destained by washing overnight in a solution of 1 % methanol and 7 % acetic acid. A densiometric scan of all samples were then made with a Gilford Linear Transport Scanner, model 2520-H.

RESULTS

Purification of MIF by CPG-Adsorption Chromatography

Two previous reports (11,86) describing the ability of CPG to purify some proteins, prompted this investigation concerning its applicability towards the purification of interferons. Binding of MIF did indeed occur, and, therefore, in order to optimize the degree of specificity or purity, a variety of elution conditions were tested.

The first elution strategy is depicted in Figure 1. Three and two-tenths million units of crude MIF (1.6×10^4 units/ml), containing 128 mg protein (0.64 mg/ml), or a specific activity of 2.5×10^4 units/mg, were loaded onto a 0.9 x 12 cm column of CPG, equilibrated in PBS. Subsequent to washing the column with PBS and 10 mM gly-HCl, pH 2.5, the interferon, 2.0×10^6 units (or 63 % of the starting material), was eluted with 100 mM KCl-HCl, pH 2.0. The peak fraction (number 43) contained 1.0×10^5 units of activity, and 0.068 mg protein, which corresponds to a specific activity of 1.5×10^6 units/mg, and represents an 60-fold purification. Thus, unlike what has been described for other proteins (11,86), interferons, bound to CPG, elute quite specifically under acid conditions.

The data presented in Figure 2 demonstrate that MIF preparations also contain other CPG-binding proteins which elute under alkali conditions. Six and three-tenths million units of crude MIF (6.3×10^4 units/ml) were loaded onto a 0.9 x 12 cm column of CPG, equilibrated in PBS. Subsequent to washing the column in PBS, 10 mM solutions of Tris-HCl, pH 8.0 and 8.5, and gly-HCl, pH 2.5, the interferon, 4×10^6 units (or 64 % of the starting material) was eluted with 100 mM KCl-HCl, pH 2.0. From the total protein recovered, 93 %, 6.9 %, and 0.3 % were found in the fractions containing PBS, 10 mM Tris-HCl, pH 8.0 - 8.5, and 100 mM KCl-HCl, pH 2.0, respectively.

Although an appreciable amount of protein eluted under alkali conditions, no interferon was detected. Thus a clear separation of CPG-bound proteins can be made depending upon the conditions of pH.

As KCl is a chaotrophic salt, glycine-HCl (gly-HCl) was examined as an alternative buffer in an attempt to improve the degree of purification

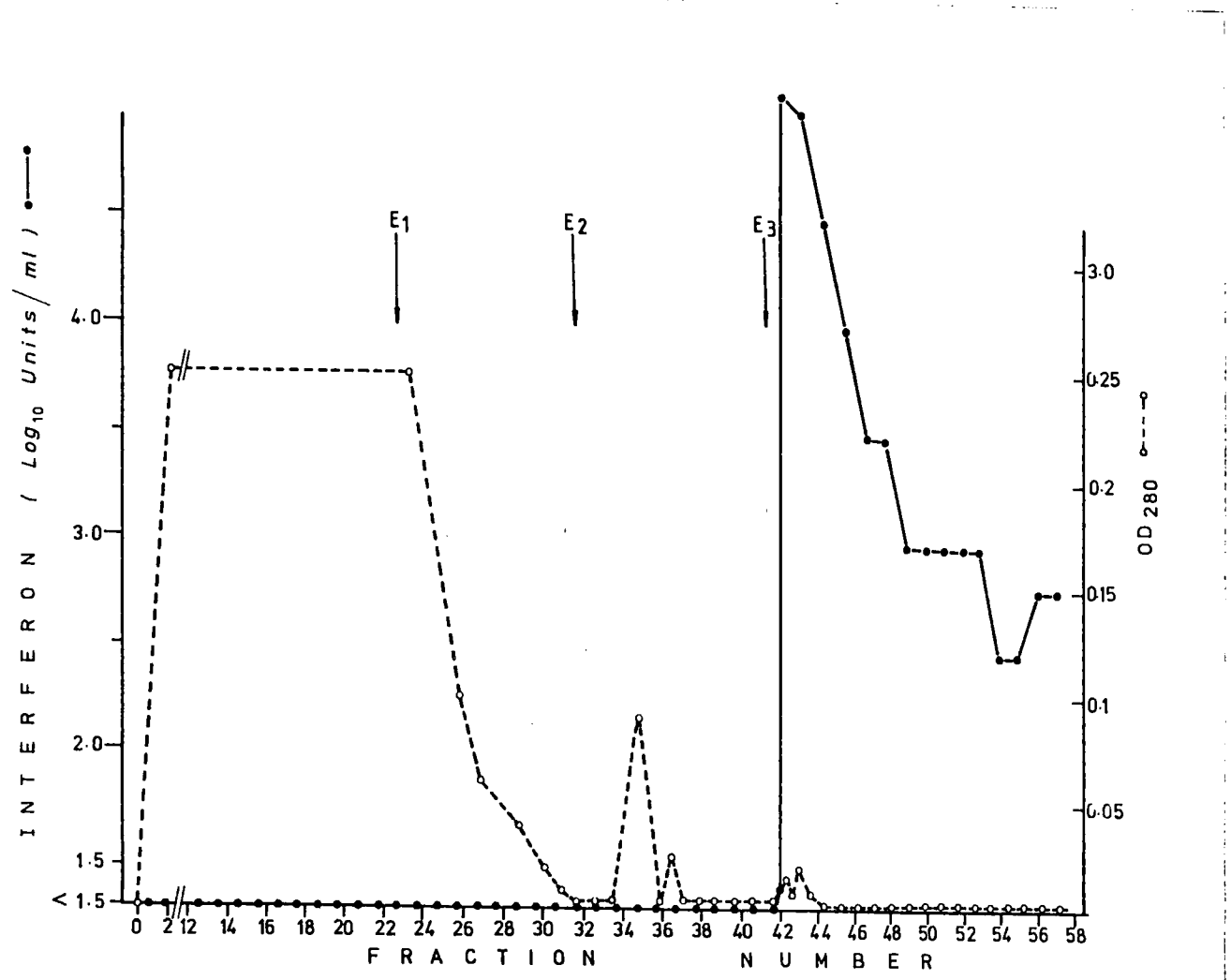

FIGURE 1. CPG-adsorption chromatography of crude MIF with acid eluants. Two-hundred ml of crude MIF were loaded onto a 0.9 x 12 cm column of CPG, equilibrated in PBS. The flow rate was 10 ml/hr and 6.7 ml/fraction were colleted. The eluant PBS is denoted as E_1, 10 mM gly-HCl, pH 2.5, as E_2 and 100 mM KCl-HCl, pH 2.0, as E_3. The effluent was continuously monitored at 280 nm (o---o) and the fractions were assayed for interferon (●——●).

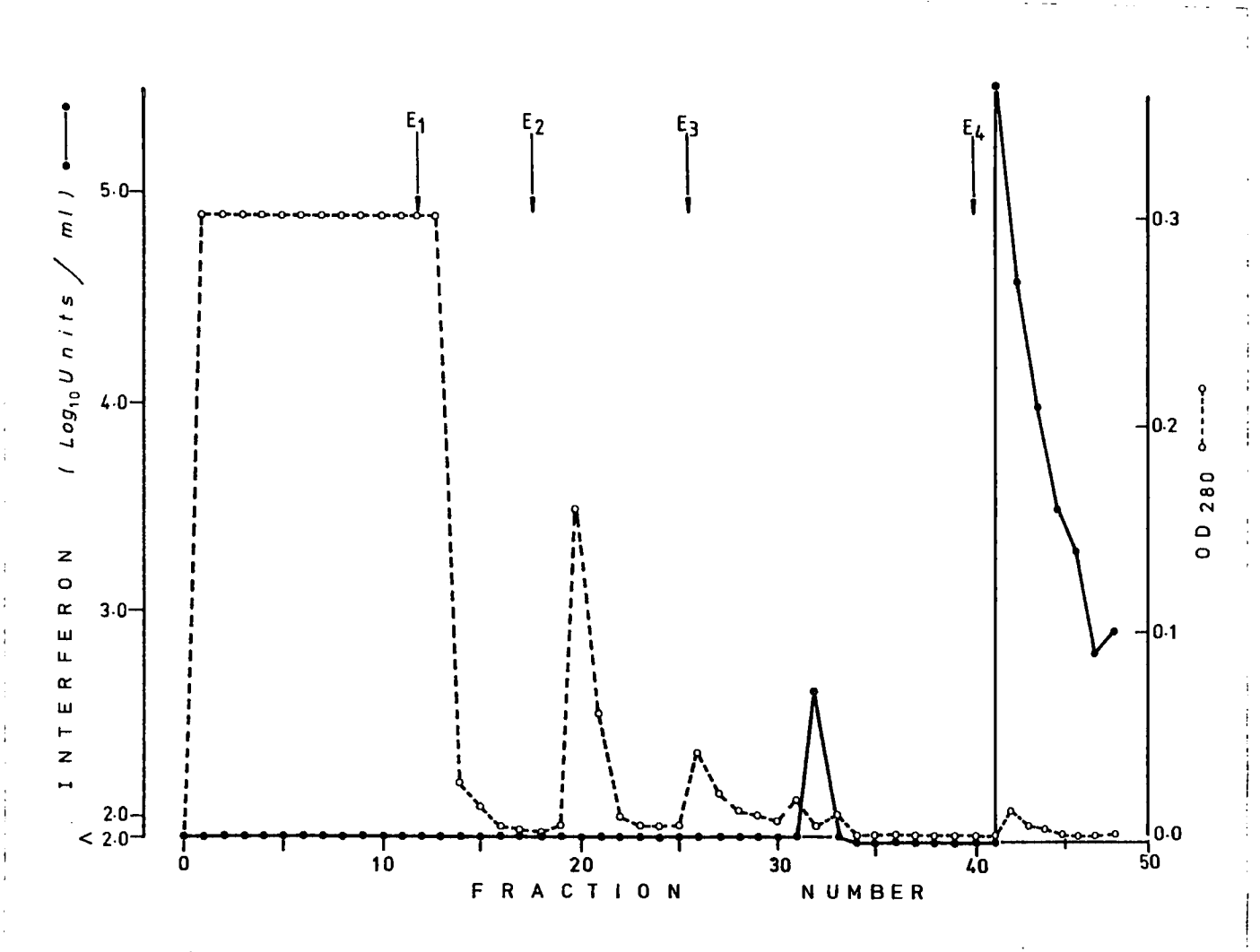

FIGURE 2. CPG-adsorption chromatography of MIF with alkali and acid eluants. One-hundred ml of crude MIF were loaded onto a 0.9 x 12 cm column of CPG, equilibrated in PBS. The flow rate was 10 ml/hr, and 6.7 ml/ fraction were collected. The eluant PBS is denoted as E_1, 10 mM Tris-HCl, pH 8.0 and 8.5, as E_2, 10 mM gly-HCl, pH 2.5, as E_3, and 100 mM KCl-HCl, pH 2.0, as E_4. The effluent was continuously monitored at 280 nm (o---o) and the fractions were assayed for interferon (●——●).

and/or yields. Furthermore, as shown in Figure 3, acid-elutable proteins could be separated on the basis of pH. Four million units of crude (4.0×10^4 units/ml), containing 100 mg protein (1.0 mg/ml), or a specific activity of 4.0×10^4 units/mg, were loaded onto a 0.5 x 5.0 cm column of CPG, equilibrated in PBS. After washing the column in PBS and 100 mM gly-HCl, pH 3.5, the interferon, 9.7×10^5 units (or 24 % of the starting material) was eluted with 100 mM gly-HCl, pH 2.0. The peak fractions (number 54-55) contained 2.5×10^5 units of activity and 0.142 mg protein, which corresponds to a specific activity of 1.8×10^6 units/mg, and represents a 45-fold purification. Although many acid-bound proteins eluted at pH 3.5, the bulk of interferon's activity eluted within a narrow range in pH (2.3 - 2.7). However, in terms of degree of purification, as compared to the elution procedure described in Figure 1, no significant improvement was noted with this elution procedure.

The elution profile of 1°-MIF, on CPG, was also examined to determine whether the purity of the final product could be increased if during ammonium sulfate precipitation some proteins were removed which would otherwise co-elute with MIF. As shown in Figure 4, 4.5×10^6 units of 1°-MIF (2.5×10^5 units/ml), containing 5.76 mg protein (0.32 mg/ml), or a specific activity of 7.8×10^5 units/mg, were loaded onto a 0.5 x 5.0 cm column of CPG, equilibrated in PBS. After washing the column of PBS and 100 mM gly-HCl, pH 3.5, the interferon, 2.5×10^6 units (or 55 % of the starting material), was eluted with 100 mM gly-HCl, pH 2.0. The peak fraction (number 43) contained 5.0×10^5 units of activity and 0.01 mg protein, which corresponds to a specific activity of 5.0×10^7 units/mg, and represents a 64-fold purification. The bulk of interferon's activity eluted between pH 2.6 - 3.2

One may conclude therefore that, if the starting material, as a result of ammonium sulfate precipitation, is of a greater purity, the specific activity, of the CPG-eluted fractions, is proportionately increased.

Mechanism of Binding MIF to CPG

Mouse and human fibroblast interferons are the first proteins reported to elute from CPG under acidic conditions (43). Thus far, the phenomenon of binding has been attributed to the beads' repeating silanol struc-

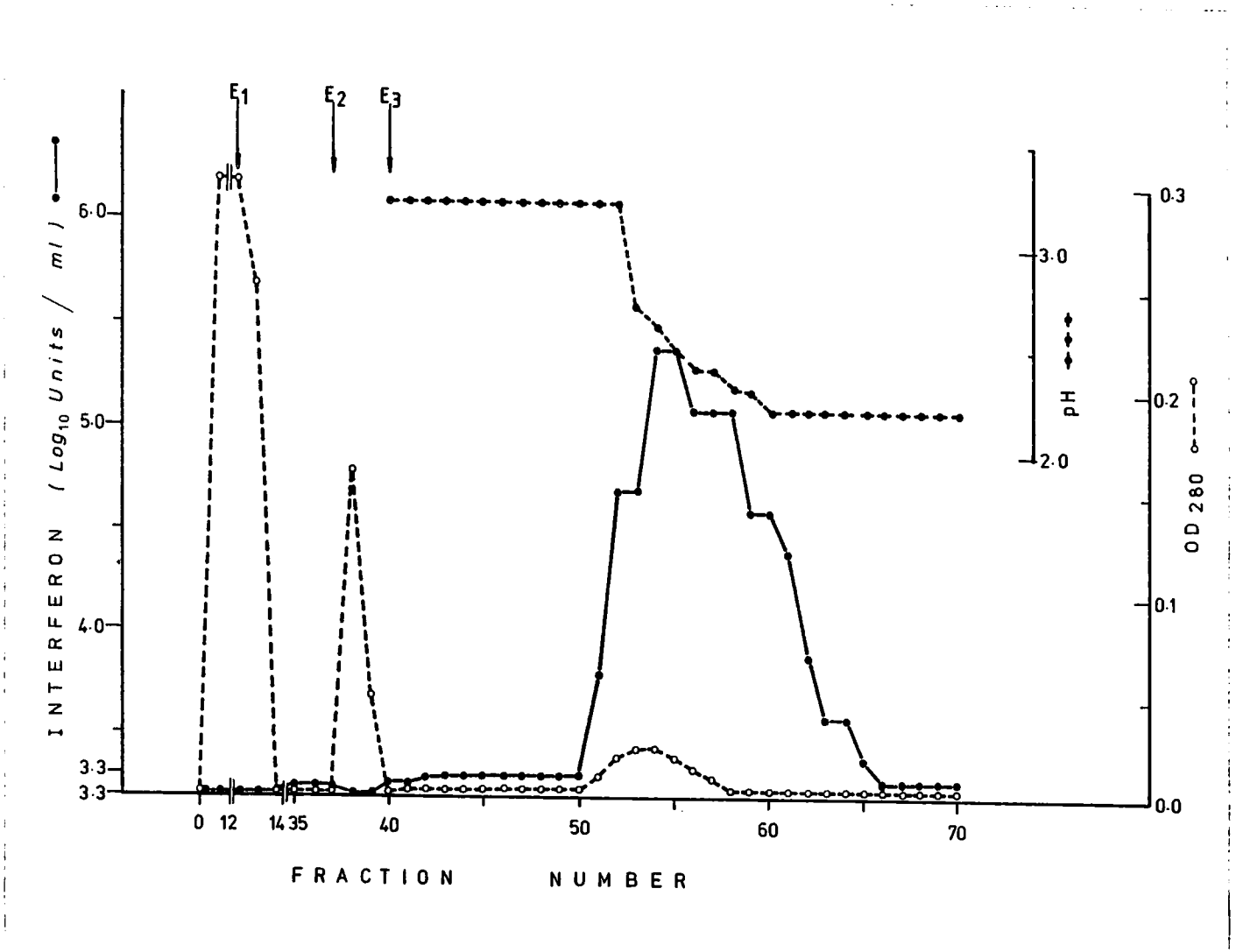

FIGURE 3. CPG-adsorption chromatography of MIF, acid pH elution profile. One-hundred ml of crude MIF were loaded onto a 0.5 x 5.0 cm column of CPG, equilibrated in PBS. The flow rate was 10 ml/hr. Fractions 1-39 contained 6.7 ml/fraction, and 40-70, 1 ml/fraction. The eluant PBS is denoted as E_1, 10 mM gly-HCl, pH 3.5, as E_2, and 100 mM gly-HCl, pH 2.0, as E_3. The effluent was continuously monitored at 280 nm (o---o), the fractions were assayed for interferon (●——●), and the pH was determined for fractions 40-70 (●-●-●).

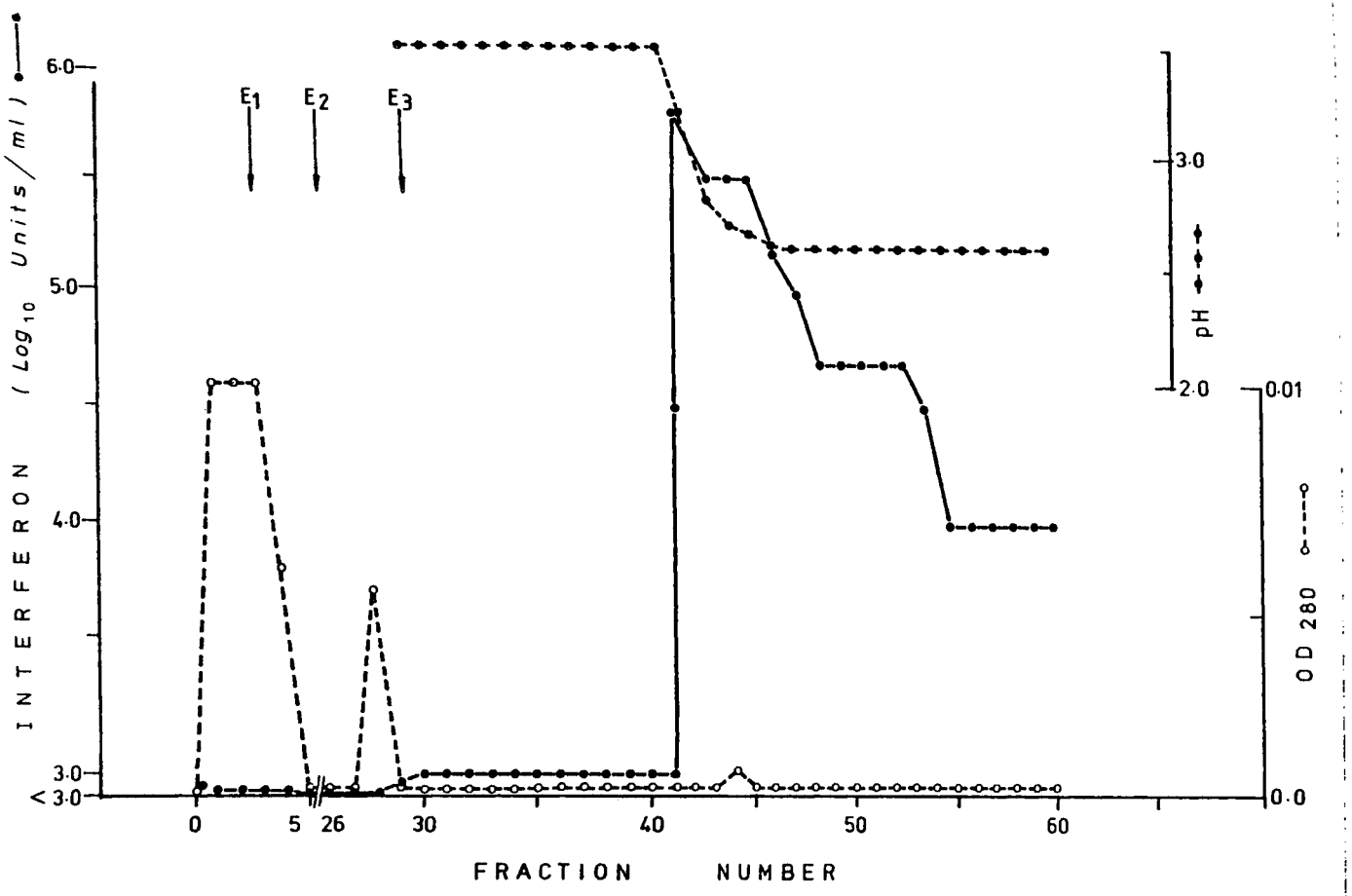

FIGURE 4. CPG-adsorption chromatography of 1°-MIF, acid pH elution profile. Eighteen ml of 1°-MIF were loaded onto a 0.5 x 5.0 cm column of CPG, equilibrated in PBS. The flow rate was 10 ml/hr. Fractions 1-29 contained 6.7 ml/fraction, and fractions 30-60, 1 ml/fraction. The eluant PBS is denoted as E_1, 100 mM gly-HCl, pH 3.5, as E_2, and 100 mM gly-HCl, pH 2.0, as E_3. The effluent was continuously monitored at 280 nm (o---o), the fractions were assayed for interferon (●——●), and the pH (●-●-●).

ture (86). However, clearly more than one binding mechanism must occur since at least two distinct classes of proteins (i.e. interferons and CPG-binding proteins other than interferons) are resolved on CPG. This section provides evidence for the role of lysine and arginine, and perhaps more nonspecifically hydrophobicity, in the mechanism of interferon binding to CPG.

Chemically accessible residues of lysine or arginine have been reported to become reversibly blocked when proteins are treated with citraconic anhydride (41). As shown in Figure 5, MIF treated with this reagent (CT-MIF) did not bind to CPG.

When 2.5×10^5 units of CT-MIF were loaded onto a 1.0 x 1.5 cm column of CPG, 2.1×10^5 units, or 84 % of the starting material, was detected in the drop-through fractions. Fractions collected from the acid-wash contained no detectable activity. The converse was observed when 3.2×10^5 units of MIF were loaded, and 1.6×10^5 units, or 50 % of the applied material was detected in the acid-wash.

These results indicate that MIF, pre-treated with citraconic anhydride, no longer binds to CPG, and that, presumably, this is due to the required lysine or arginine residue becoming temporarily blocked.

Further proof for the role of lysine or arginine in MIF-CPG interactions is shown in Figure 6. When 1.6 absorbance units of poly-l-lysine was applied to a 1.0 x 3.0 cm column of CPG, 0.9 units (or 56 % of the starting material) and 0.8 units (or 50 %) were eluted with PBS and 400 mM gly-HCl, pH 2.0, respectively. None of the polyamino acid was observed in the 10 mM Tris-HCl, pH 8.9, wash. Thus similar to MIF, the poly-l-lysine which did bind to CPG eluted under acidic, and not under alkali conditions.

A different elution profile was obtained with poly-l-arginine. When 3.0 absorbance units were loaded, 0.89 units (or 30 % of the starting material) passed unbound through the column. Two peaks (0.16 units or 5.3 % and 0.26 or 8.7 % of the starting material) were detected in the alkali-wash, while 1.84 units or 61 % of the applied material was found in the acid-wash. Thus, although the bulk of the poly-l-arginine eluted at acid conditions, unlike poly-l-lysine, some eluted, in a bi-modal fashion, at high pH.

In addition to silanol groups, CPG, at its surface, contains a significant concentration of boron (136). Thus the beads contain both hydroxyl groups, and <u>via</u> permeations of B_2O_3 groups, Lewis acid sites. Conceivably,

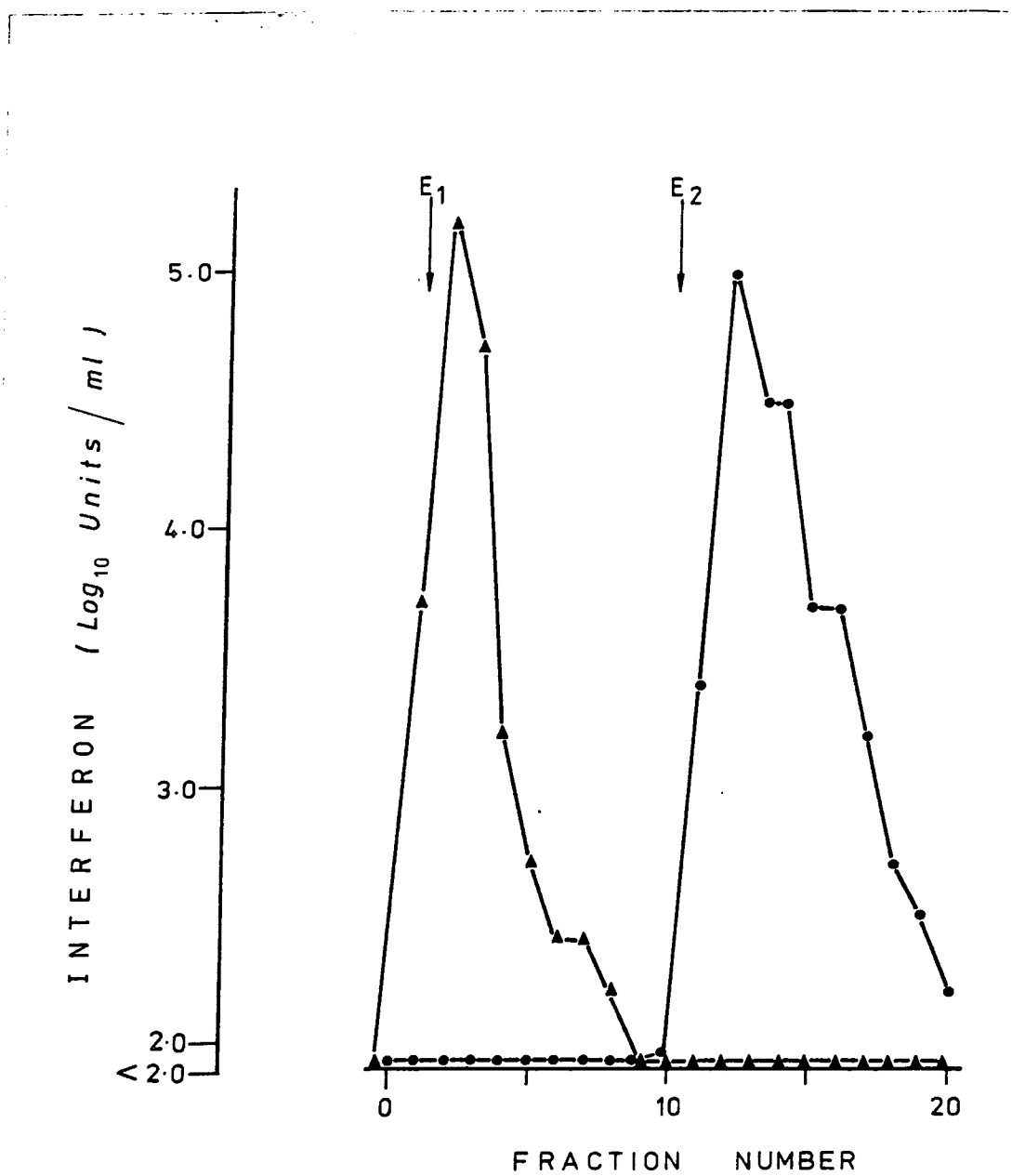

FIGURE 5. CPG-adsorption chromatography of citraconylated MIF (CT-MIF). A 1.0 x 1.5 cm column of CPG, equilibrated in PBS, was loaded with either 1 ml CT-MIF (▲——▲) or MIF (●——●). Ten 1-ml fractions, containing 0.1 ml HPP (22.5 mg/ml) were collected. The eluant PBS is denoted as E_1, and 400 mM gly-HCl, pH 2.0, as E_2.

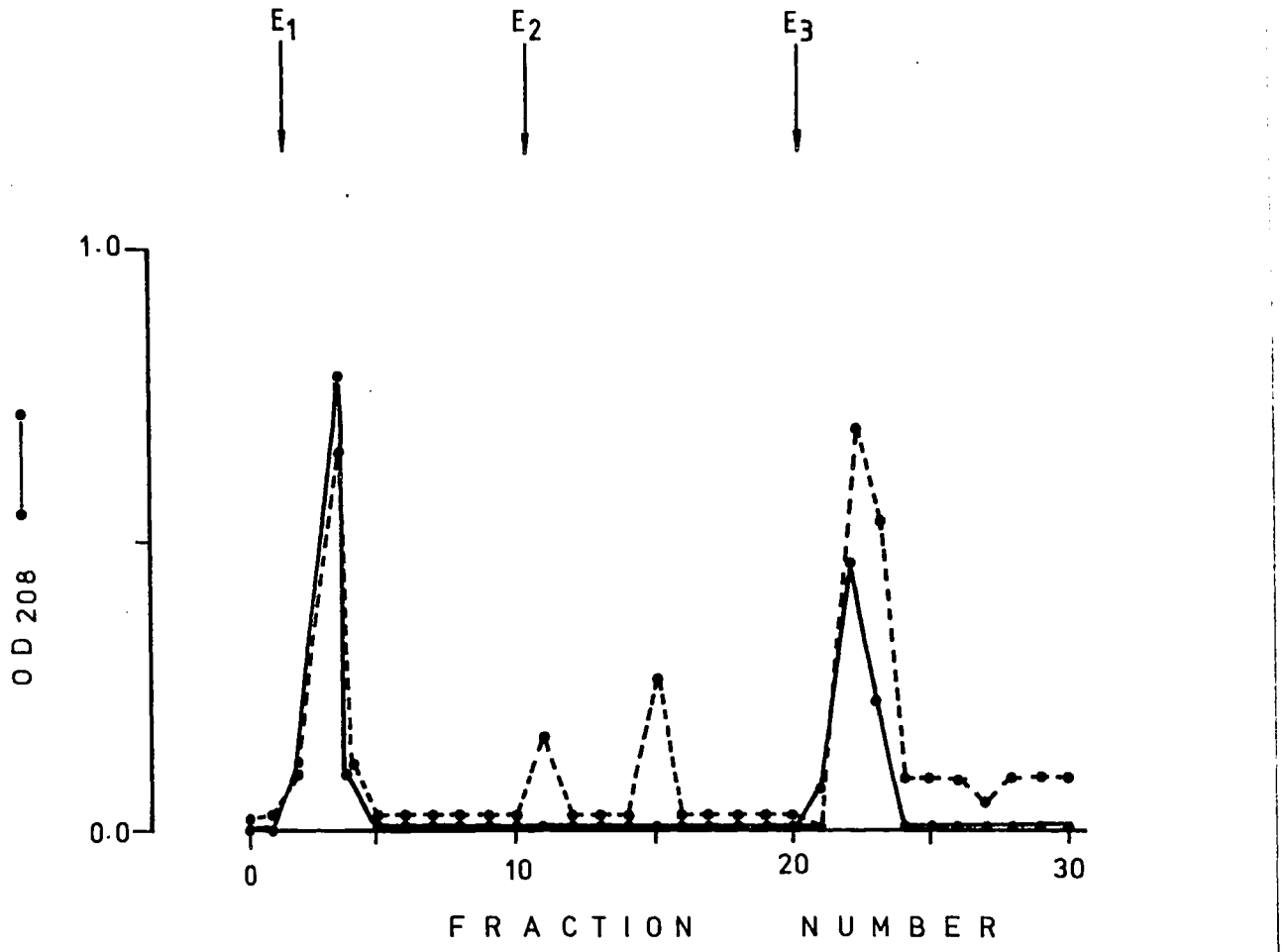

FIGURE 6. CPG-adsorption chromatography of poly-l-lysine and poly-l-arginine. One ml of poly-l-lysine (100 µg, OD_{208} = 1.6 absorbance units), and poly-l-arginine (100 µg, OD_{208} = 3.0 absorbance units), in PBS, were loaded onto a 1.0 x 3.0 cm column of CPG. Ten - 1 ml fractions were collected. The eluant PBS is denoted as E_1, 10 mM Tris-HCl, pH 8.9, as E_2 and 400 mM gly-HCl, pH 2.0, as E_3. The absorbance of poly-l-lysine (●——●) and poly-l-arginine (●---●) fractions were determined at 208 nm.

although the pH value for the ε-aminogroup of lysine (10.53) and the guanidinium group of arginine (12.48) is too high for Lewis acid-base interactions to occur, binding might occur *via* the terminal primary amine group of arginine. Thus, in order to determine whether mouse interferon binds to CPG on the basis of charge or Lewis interactions, another polymer, ZrOH (63), which contains hydroxyl groups but is free of boron, was tested.

As indicated in Table 1, MIF adsorbs to ZrOH both in a column, and, to a lesser extent, batch procedure. In the latter, when 1.6×10^6 units of crude MIF (1.6×10^4 units/ml), containing 100 mg protein (1 mg/ml), or a specific activity of 1.6×10^4 units/mg, was treated with ZrOH, 3.0×10^5 units (or 18 % of the applied activity) was recovered in the PBS-wash. No activity was detected in the acid-wash with 400 mM gly-HCl, pH 3.5. However, when the pH was reduced to 2.0, 1.6×10^5 units (or 10 % of the applied activity), and 0.4 mg protein was recovered, which corresponds to a specific activity of 4×10^5 units/mg, and represents a 25-fold purification.

Similarly, when 1.0×10^5 units of crude MIF (1.0×10^4 units/ml), containing 2.7 mg protein (0.27 mg/ml), or a specific activity of 3.7×10^4 units/mg, were loaded onto a 0.5 x 5.0 cm column of ZrOH, no activity was detected in either the PBS or 400 mM gly-HCl, pH 3.5, washes. Fractions obtained with 400 mM gly-HCl, pH 2.0, contained a total of 1.25×10^5 units of activity (or 125 % of the applied material). The peak fraction had 5×10^4 units of activity, and 0.044 mg protein, which corresponds to a specific activity of 1.1×10^6 units/mg, and represents a 30-fold purification.

Thus MIF binds to a polymer containing a high quantity of hydroxyl groups, but none of the known impurities found in CPG. Furthermore, by virtue of the degree of purification obtained with ZrOH, one may conclude that interferon binds to CPG and ZrOH with about the same selectivity.

Since many hydrophobic compounds readily adsorb to SiO_2 groups (44), the buffer PBS containing ethylene glycol and NaCl was tested as an eluant. As indicated in Figure 7, when 8.0×10^5 units (1.6×10^5 units/ml) crude MIF were loaded onto a 0.5 x 5.0 cm column of CPG, 6.2×10^3 units (or 0.78 % of the starting material) eluted unbound 2.2×10^5 units (or 28 %) eluted with PBS containing 10 M ethylene glycol and 1 M NaCl (EG-NaCl), pH 7.2, and 1.0×10^3 units (or 0.13 %) eluted with 400 mM gly-HCl, pH 2.0. No activity was detected from fractions containing 10 mM Tris-HCl, pH 8.9.

TABLE 1. ZrOH Adsorption of Crude MIF

Description	Total units recovered (log_{10})	% Recovery[(a)]
Batch :		
After loading	4.7	3.1
First PBS wash	5.2	10.0
Second PBS wash	4.5	2.0
Third PBS wash	4.5	2.0
Fourth PBS wash	4.2	1.0
Fifth PBS wash	3.7	0.3
400 mM gly-HCl, pH 3.5	< 3.0	< 0.06
400 mM gly-HCl, pH 2.0	5.2	10.0
Column :		
After loading	< 1.6	< 0.04
First PBS wash	< 1.6	< 0.04
Second PBS wash	< 1.6	< 0.04
Third PBS wash	< 1.6	< 0.04
Fourth PBS wash	< 1.6	< 0.04
400 mM gly-HCl, pH 3.5	< 1.6	< 0.04
400 mM gly-HCl, pH 2.0	5.1	125.0

(a) Starting material for the batch procedure contained 1.6×10^6 units (1.6×10^4 units/ml), while for the column 1.0×10^5 units (1.0×10^4 units/ml).

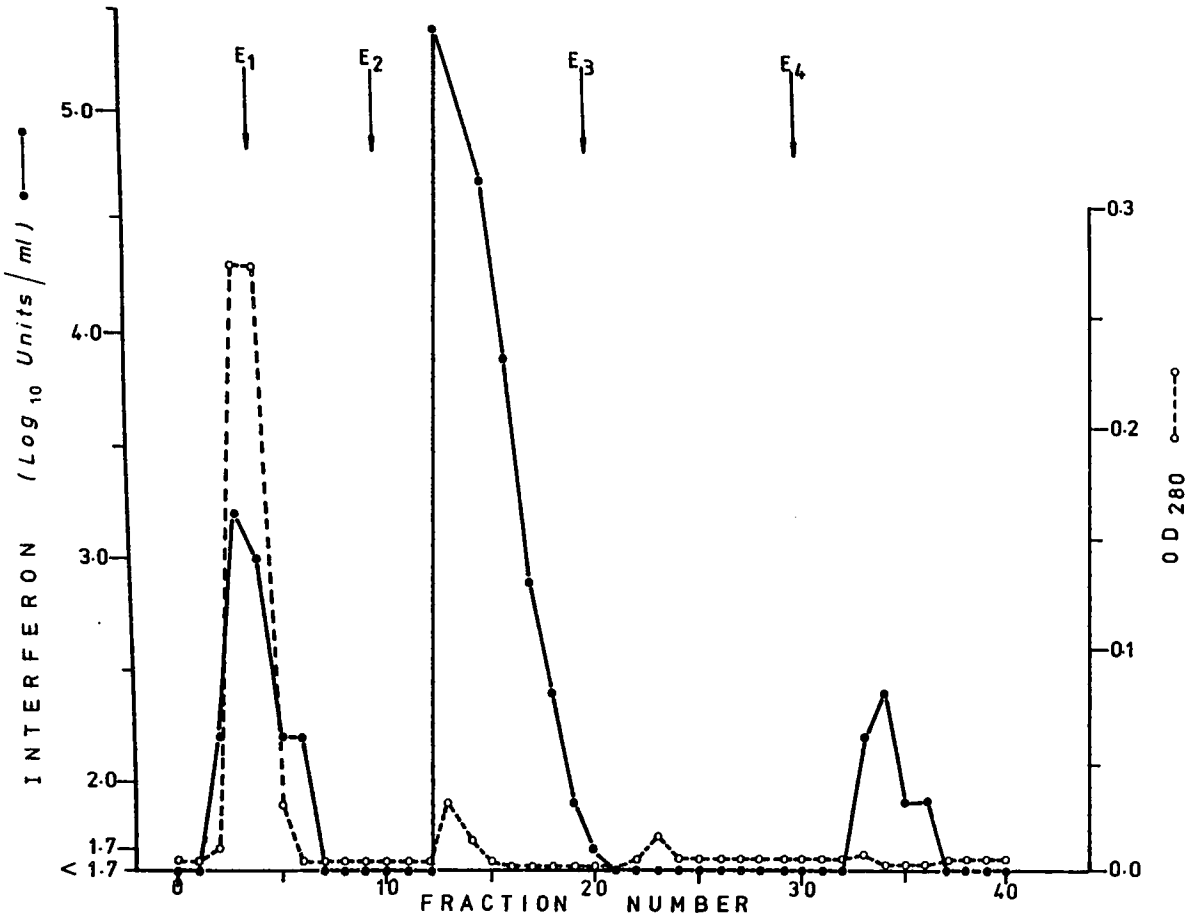

FIGURE 7. CPG-adsorption chromatography of MIF with a hydrophobic eluant. Five ml of crude MIF were loaded onto a 0.5 x 5.0 cm column of CPG, equilibrated in PBS. The flow rate was 10 ml/hr, and 2 ml/fraction, containing 0.1 ml HPP (22.5 mg/ml), were collected. The eluant PBS is denoted as E_1, PBS containing 10 M ethylene glycol and 1 M NaCl, as E_2, 10 mM Tris-HCl, pH 8.9, as E_3, and 400 mM gly-HCl, pH 2.0, as E_4. The effluent was continuously monitored at 280 nm (o---o) and fractions (E_2, E_3, and E_4 were first dialyzed against PBS), were assayed for interferon (●——●).

From the total protein recovered, 93 %, 4.2 %, 2.2 % and 0.13 % were found in the fractions containing PBS, PBS + EG-NaCl, 10 mM Tris-HCl, pH 8.9, and 400 mM gly-HCl, pH 2.0, respectively.

Organomercurial Affinity Chromatography

The recognition that many proteins, via the thiol groups of cysteine, covalently bind to such heavy metals as mercury to form mercaptides, has prompted the use of organomercurials as an affinity ligand (13,90,109,115). The ability of MIF to bind to one such ligand, Agarose-p-aminophenyl mercuric acetate (Hg-agarose) was also tested.

As indicated in Figure 8, MIF both binds to and elutes from this ligand. Two-hundred and fifty-thousand units of 1°-MIF (2.5×10^5 units/ml) were loaded onto a 0.5 x 5.0 cm column of Hg-agarose, equilibrated in 100 mM Na-PB, pH 8.0. The column was first washed with equilibration buffer, then washed with 50 mM NaAc, pH 5.0, to remove any nonspecifically bound proteins (i.e. those which did not form covalent bonds), and finally washed with the same buffer containing first 0.5 mM, and later, 2.5 mM cysteine. No activity was detected in either the drop-through or 50 mM NaAc, pH 5.0, washes. However, a total of 1.0×10^4 units of interferon (or 4 % of the starting material), and 1.0×10^5 units (or 40 % of the applied material) were detected in the eluates containing 0.5 mM and 2.5 mM cysteine, respectively.

The protein elution profile (Figure 8), measured at 280 nm, indicates that the degree of purification by this procedure was not significant. However, a clear resolution was witnessed between those proteins which contained accessible cysteine residues and those proteins which did not contain accessible thiol groups. MIF was amongst the former.

Hydrophobic Chromatography of 1°-MIF with AFFI-Gel 202

The purification of crude MIF on AFFI-Gel 202 has been previously described (33). This method has now been applied in the purification of 1°-MIF and the results are depicted in Figure 9 and summarized in Table 2.

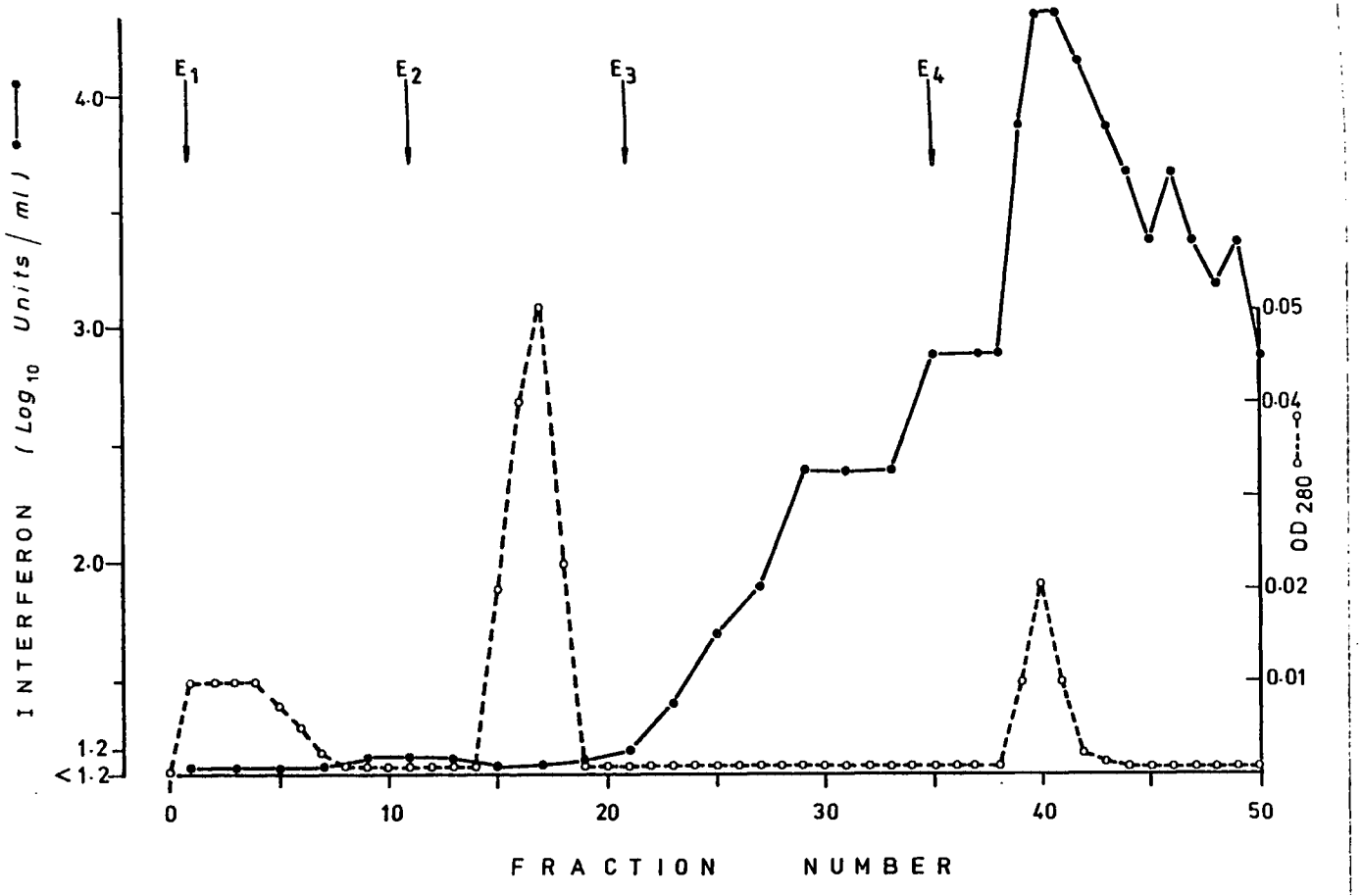

FIGURE 8. Organomercurial affinity chromatography with Hg-agarose. One ml of 1°-MIF was loaded onto a 0.5 x 5.0 column of Hg-agarose, equilibrated in 100 mM Na-PB, pH 8.0. The flow rate was 10 ml/hr, and 1 ml/fraction were collected. The eluant 100 mM Na-PB, pH 8.0, is denoted as E_1, 50 mM NaAc, pH 5.0, as E_2, and 50 mM NaAc, pH 5.0, containing 0.5 mM and 2.5 mM cysteine as E_3 and E_4, respectively. The effluent was continuously monitored at 280 nm (o---o) and between fractions 1-36 only the odd-numbered fractions, and between fractions 37-50 all fractions, were assayed for interferon (●——●).

Three and five-tenths million units of 1°-MIF (1.6×10^5 units/ml), containing 12.2 mg protein (0.55 mg/ml), or a specific activity of 2.9×10^5 units/mg, were loaded onto a 0.9 x 12 cm column of AFFI-Gel 202, equilibration in 50 mM NaAc, pH 5.0. No activity was detected during this buffer wash, while 2.5×10^5 units (or 7 % of the starting material) eluted with 20 mM Na-PB, pH 7.2. The remainder of the recoverable activity, 1.28×10^6 units (or 37 %) eluted with PBS containing 350 mM NaCl, pH 7.2. With this eluant, one peak fraction (number 55) had 3.2×10^5 units, and 0.1 mg protein, which corresponds to a specific activity of 3.2×10^6 units/mg, and represents a 14-fold purification.

Gel Filtration Chromatography of 1°-MIF with Ultrogel AcA 54

Gel filtration, with Ultrogel AcA 54, was also tested as a means of further purifying MIF. As indicated in Figure 10, and summarized in Table 2, 2.6×10^7 units of 1°-MIF (2.0×10^6 units/ml), containing 26 mg protein (2 mg/ml), or a specific activity of 1×10^6 units/mg, were loaded onto a 1.6 x 63 cm column of Ultrogel AcA 54, equilibrated in 50 mM K-PB, pH 5.9. Although two peaks of protein were observed, antiviral activity was detected only in the second. The total activity recovered under the curve was 3.2×10^6 units, or 12 % of the starting material. The peak fraction (number 56) contained 6.3×10^5 units , and 0.021 mg protein, which corresponds to a specific activity of 3.0×10^7 units/mg, and represents a 30-fold purification.

The molecular weights of 2°-MIF, scheme 2, was determined by loading 3.0×10^7 units of interferon onto a similar size column of Ultrogel AcA 54, equilibrated in 50 mM K-PB, pH 5.9. As depicted in Figure 11, the molecular weights of the eluted peak activity, which represents 21 % (6.4×10^6 units of activity) of the applied material, was between 11,000 - 12,000 daltons. The total activity recovered was 29 % or 8.6×10^6 units.

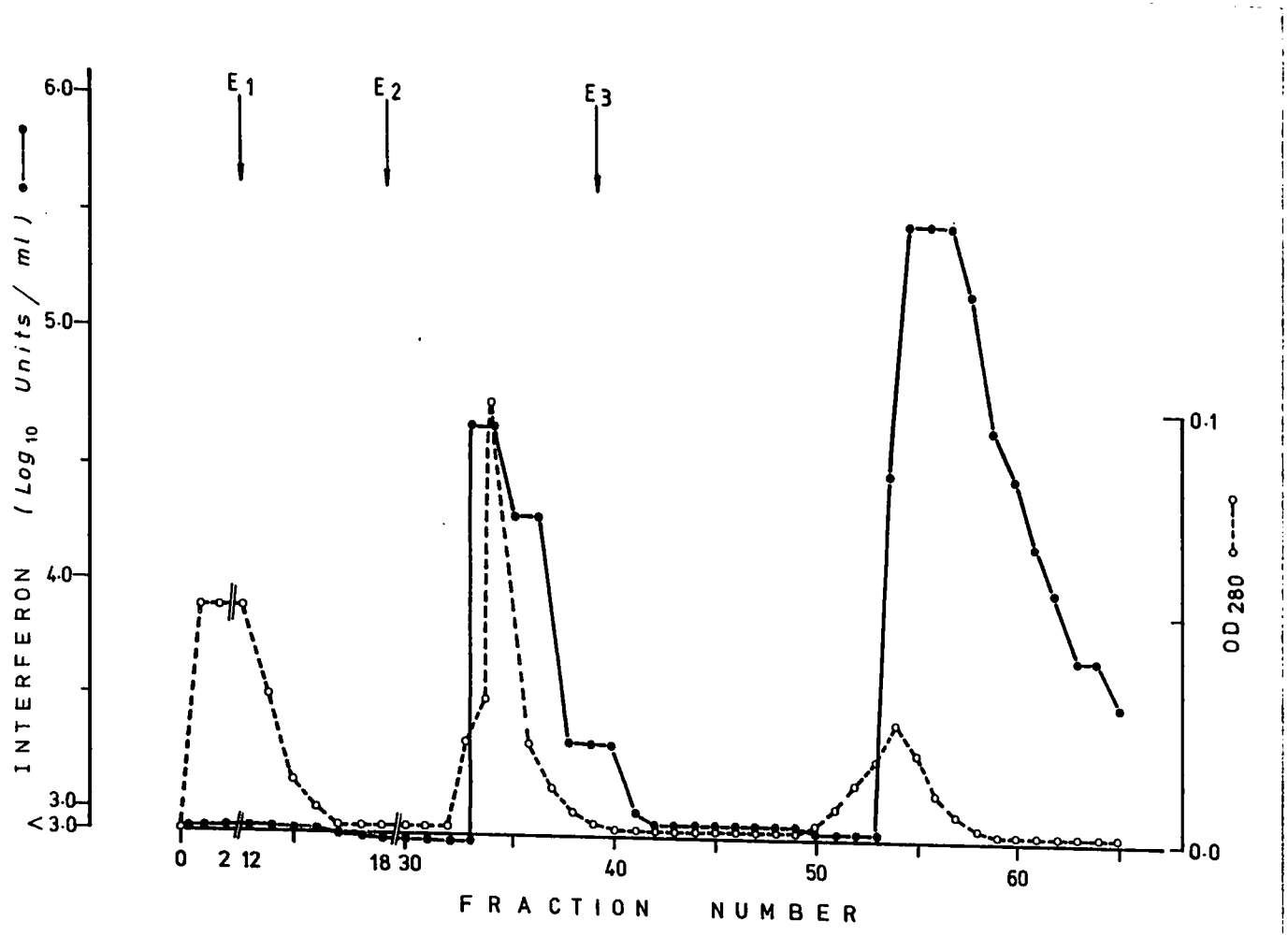

FIGURE 9. Hydrophobic chromatography of 1°-MIF with AFFI-Gel 202. Twenty-two ml of 1°-MIF were loaded onto a 0.9 x 12 cm column of AFFI-Gel 202, equilibrated in 50 mM NaAc, pH 5.0. The flow rate was 10 ml/hr. Fractions 1-39 contained 2 ml/fraction and fractions 40-70, 1 ml/fraction. The eluant 50 mM NaAc, pH 5.0, is denoted as E_1, 20 mM Na-PB, pH 7.2, as E_2, and PBS containing 350 mM NaCl, pH 7.2, as E_3. The effluent was continuously monitored at 280 nm (o---o), and the fractions were assayed for interferon (●——●).

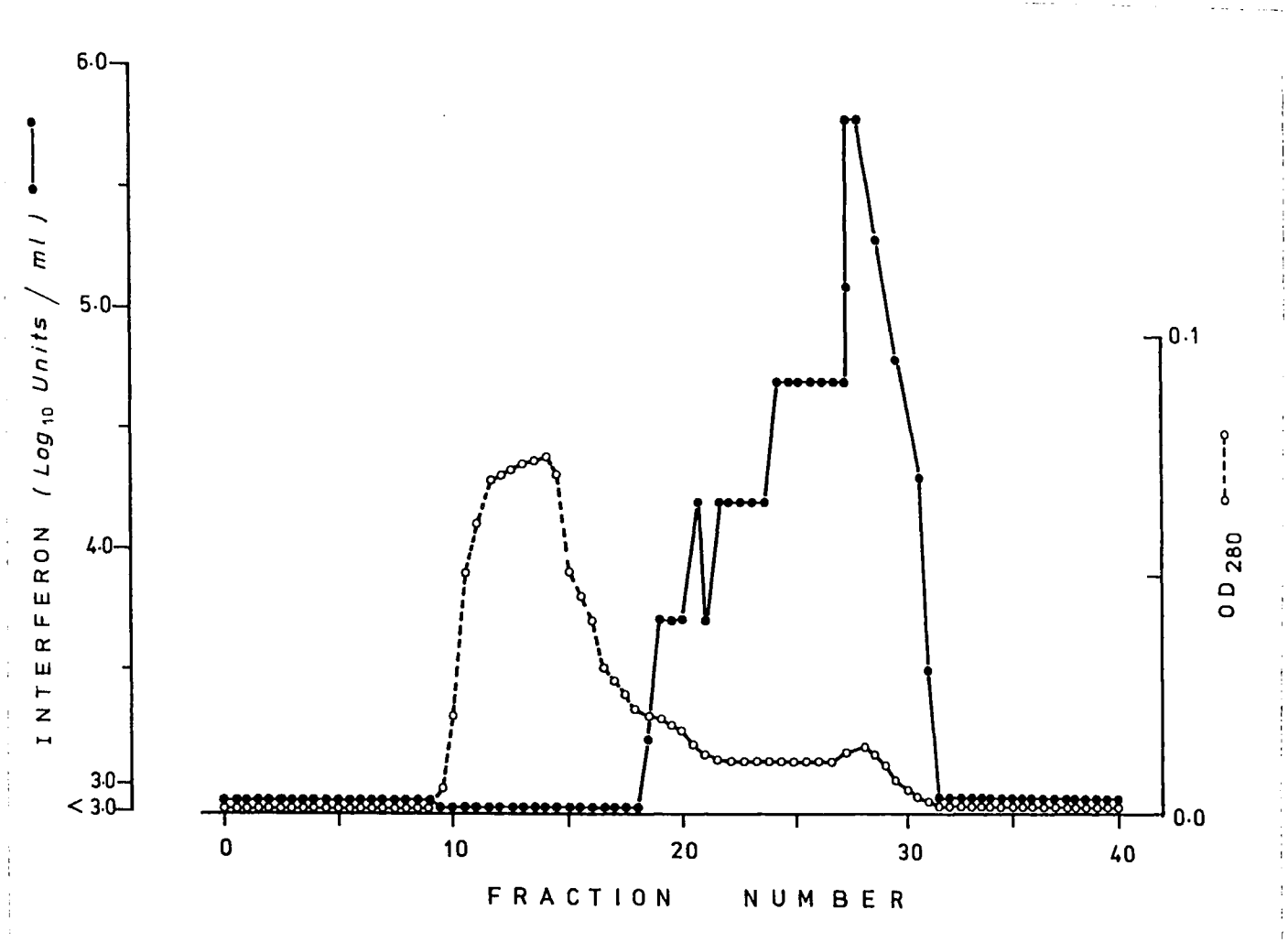

FIGURE 10. Gel filtration chromatography of 1°-MIF with Ultrogel AcA 54. Thirteen ml of 1°-MIF were loaded, after concentrating with $(NH_4)_2SO_4$, onto a 1.6 x 63 column of Ultrogel AcA 54, equilibrated in 50 mM K-PB, pH 5.9. The flow rate was 10 ml/hr and 2 ml/fraction were collected. The effluent was continuously monitored at 280 nm (o---o) and the fractions were assayed for interferon (●——●).

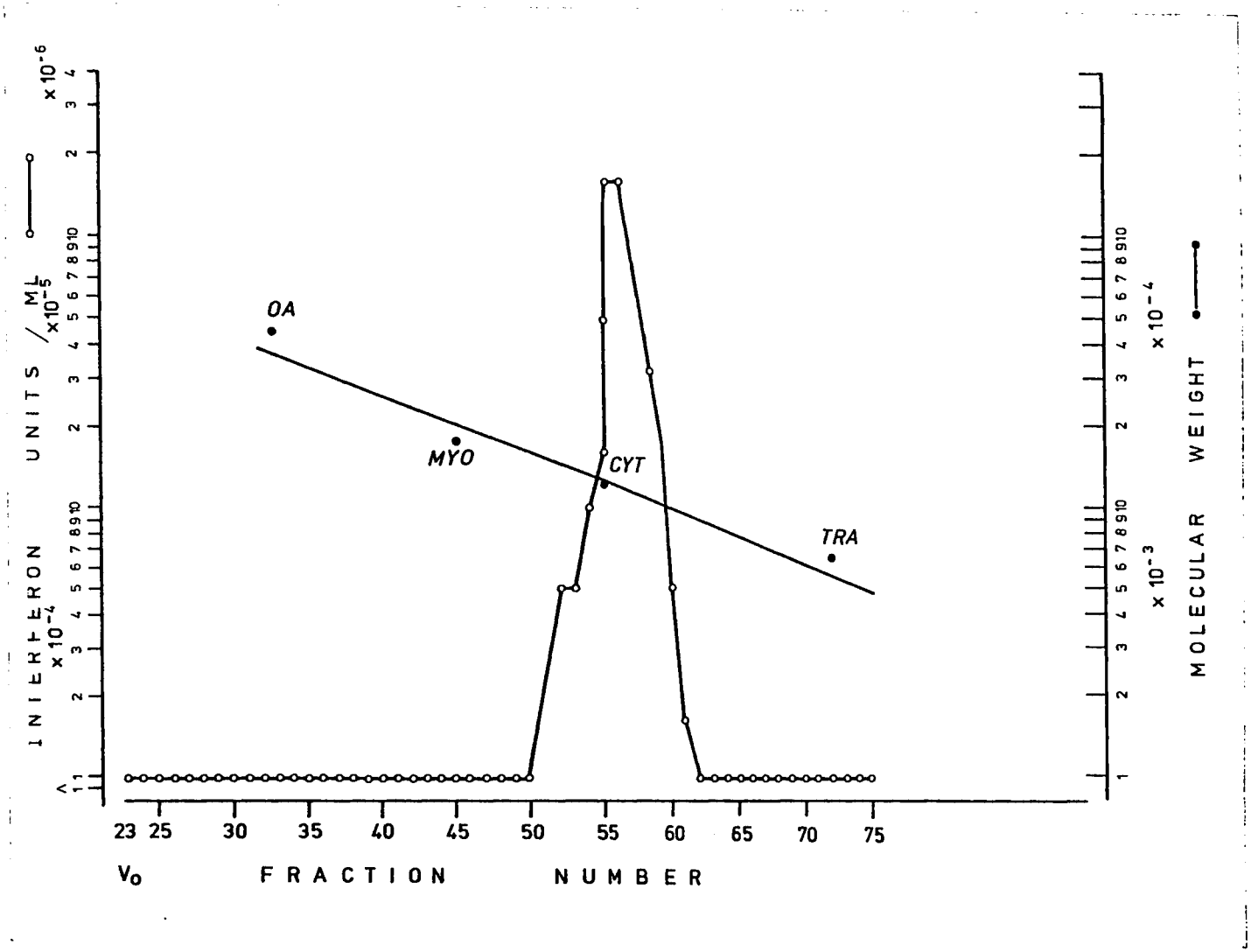

FIGURE 11. Molecular weight determination of 2°-MIF , scheme 2, by gel filtration chromatography with Ultrogel AcA 54. Eighteen ml of 1°-MIF were loaded, after concentrating with $(NH_4)_2SO_4$, onto a 1.6 x 63 cm column of Ultrogel AcA 54, equilibrated in 50 mM K-PB, pH 5.9. The flow rate was 10 ml/hr and 2 ml/fraction were collected. The fractions were assayed for interferon (●——●). The molecular weight standards were ovalbumin (OA) MW 45,000, myoglobin (MYO) MW 17,800, cytochrome C (CYT) MW 12,400 and Trasylol (TRA) MW 6,500.

Ionic Exchange Chromatography of 2°-MIF, Scheme 2, with CM-BGA

MIF was further purified by directly loading the material obtained from Ultrogel AcA 54 (2°-MIF, scheme 2) onto CM-BGA. As shown in Figure 12, 9.2×10^6 units of 2°-MIF, scheme 2, were applied to a 1.0 x 1.5 cm column of CM-BGA, equilibrated in 50 mM K-PB, pH 5.9. After washing the column in equilibration buffer, the interferon, 4.8×10^6 units (or 52 % of the loaded material), was eluted with 100 mM K-PB, pH 8.0. One peak fraction (number 6) had 2.0×10^6 units of activity, and 0.032 mg protein, which corresponds to specific activity of 6.2×10^7 units/mg, and as summarized in Table 2, with respect to the 1°-MIF employed as the starting material, represents a 238-fold purification.

Concert Chromatography

Two strategies were investigated to purify MIF by the techniques described above. Since, at low protein concentration, MIF, with respect to time, inactivates (67), and/or nonspecifically binds to many of the apparatus employed (119), both were designed to require the minimum amount of time and manipulation (e.g. dialysis and concentration). To that end, loading and elution buffers were coordinated so that preparations could be directly transferred from one column to the next.

The results from the two procedures are depicted in Table 2. Because of the limited available material, samples were not removed after each step, and the data shown are composites of several experiments. The first approach (scheme 1) consisted of three techniques : $(NH_4)_2SO_4$ precipitation (1°), hydrophobic chromatography with AFFI-Gel 202 (2°), and CPG-adsorption chromatography (3°). The data from the first two steps, 1°-2°, was described above (see hydrophobic chromatography). With the complete procedure, the starting material had 3.7×10^7 units of 1°-MIF (3.2×10^6 units/ml), containing 5.3 mg protein (0.46 mg/ml), or a specific activity of 7.0×10^6 units/mg, while the final product (3°-MIF), had a total of 2.2×10^7 units, or a 59 % recovery. The peak material contained 1.6×10^7 units, 0.02 mg protein, which corresponds to a specific activity of 8.0×10^8 units/mg, and represents a 114-fold purification.

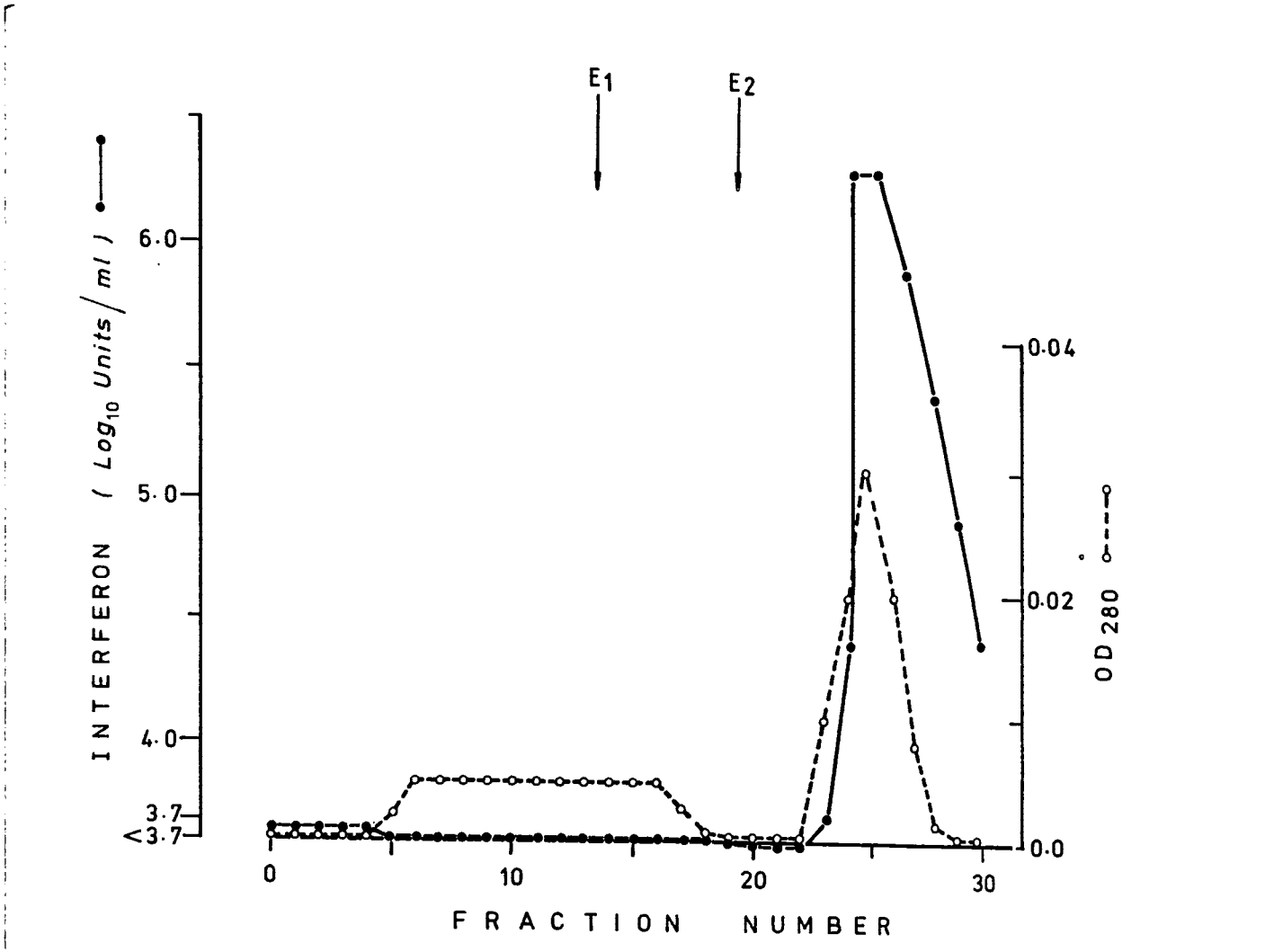

FIGURE 12. Ionic exchange chromatography of 2°-MIF scheme 2, with CM-BGA. Ten ml of 2°-MIF were loaded onto a 0.7 x 1.5 cm column of CM-BGA, equilibrated in 50 mM K-PB, pH 5.9. The flow rate was 10 ml/hr, and 1 ml/fraction were collected. The eluant 50 mM K-PB, pH 5.9, is denoted as E_1, and 100 mM K-PB, pH 8.0, as E_2. The effluent was continuously monitored at 280 nm (o---o) and the fractions were assayed for interferon (●——●).

TABLE 2. Concert Chromatographic Purification of Mouse L-929 Interferon

Procedure[a]	INPUT			OUTPUT					
	Total units applied	Total protein applied (mg)	Sp. act. (log_{10} units/mg)	Total units recovered	% recovery	Units in peak fraction	Protein in peak fraction (mg)	Sp. act. (log_{10} units/mg)	Purification factor
Scheme 1									
1° → 2°[b]	3.5×10^6	12.2	2.9×10^5	1.28×10^6	37	3.2×10^5	0.1	3.2×10^6	11
1° → 3°	3.7×10^7	5.3	7.0×10^6	2.2×10^7	59	1.6×10^7	0.02	8.0×10^8	114
Scheme 2									
1° → 2°[c]	2.6×10^7	26.0	1×10^6	3.2×10^6	12	6.3×10^5	0.021	3.0×10^7	30
1° → 3°[d]	7.5×10^6	29.0	2.6×10^5	4.8×10^6	64	2.0×10^6	0.032	6.2×10^7	238
1° → 5°	5.3×10^7	5.8	9.1×10^6	3.9×10^6	7.4	2.5×10^6	0.0067	3.7×10^8	41

(a) The steps described are as follows : scheme 1, 1°-ammonium sulfate precipitation, 2°-hydrophobic chromatography with AFFI-Gel 202, 3°-CPG-adsorption chromatography; scheme 2, 1°-ammonium sulfate precipitation, 2°-gel filtration with Ultrogel AcA 54, 3°-ionic exchange chromatography with CM-BGA, 4°-hydrophobic chromatography with AFFI-Gel 202, 5°-CPG-adsorption chromatography.

(b) See results section on hydrophobic chromatography of 1°-MIF with AFFI-Gel 202.

(c) See results section on gel filtration of 1°-MIF with Ultrogel AcA 54.

(d) See results section on ionic exchange chromatography of 2°-MIF, scheme 2, with CM-BGA.

Scheme 2 utilized five techniques : $(NH_4)_2SO_4$ precipitation (1°), gel filtration with Ultrogel AcA 54 (2°), ionic exchange chromatography with CM-BGA (3°), hydrophobic chromatography with AFFI-Gel 202 (4°), and CPG-adsorption chromatography (5°). The first three steps, 1°-2° and 1°-3°, were described above under the headings gel filtration and ionic exchange chromatography, respectively.

When steps 1°-5° were tested, of the 5.3×10^7 units of 1°-MIF (3.2×10^6 units/ml), containing 5.8 mg protein (0.36 mg/ml), or a specific activity of 9.1×10^6 units/mg, applied, a total of 3.9×10^6 units, or 7.4 % was recovered. The peak material contained 2.5×10^6 units, and 0.0067 mg protein, which corresponds to a specific activity of 3.7×10^8 units/mg, and represents a 41-fold purification.

The final material, prepared from schemes 1 and 2, were analyzed for activity (Figure 13) on preparative SDS-PAGE gels and protein content (Figure 14) on analytical SDS-PAGE gels. From the 3.2×10^4 units of scheme 1 activity applied, a total of 2.6×10^3 units or 8 % of the loaded material was recovered. Two peaks of activity were detected. The first, representing 48 % (1.25×10^3 units) of the recovered activity, had a molecular weight of between 39,000 - 44,000 daltons. The second peak, representing 30 % (8.0×10^2 units) of the recovered activity, had a molecular weight varying from 19,000 to 23,000 daltons. Scheme 1 samples analyzed on an analytical gel had two protein peaks, one with an approximate molecular weight of 30,000 daltons, the other less than 14,000 daltons.

When scheme 2 material (1.0×10^4 units of activity) was applied on preparative gels, 17.0 % or 1.7×10^3 units of activity was recovered. Only one peak of activity was detected. This represented 74 % (1.25×10^3 units of activity) of the recovered material and had a molecular ranging from 39,000 to 44,000 daltons. No protein was detected from scheme 2 material applied to the analytical gel.

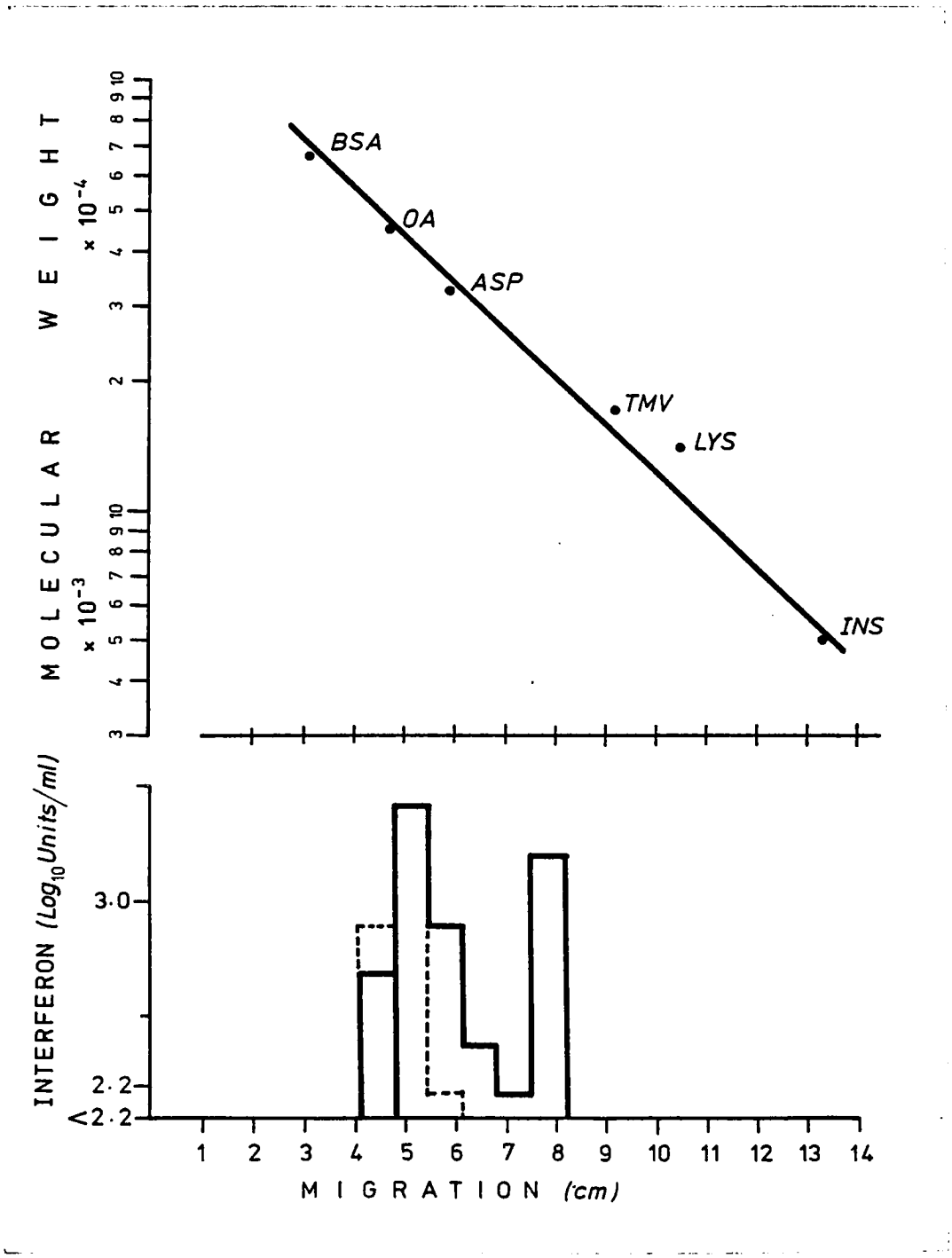

FIGURE 13. Preparative SDS-PAGE of schemes 1 and 2 MIF. Molecular weight determinations of MIF preparations obtained from concert chromatography schemes 1 (——) and 2 (---) were made by SDS-PAGE. The molecular weight standards were bovine serum albumin (BSA) MW 67,000, ovalbumin (OA) MW 45,000, asparaginase (ASP) MW 33,000, tobacco mosaic virus (TMV) MW 17,000, lysozyme (LYS) MW 14,000, and insulin (INS) MW 5,600.

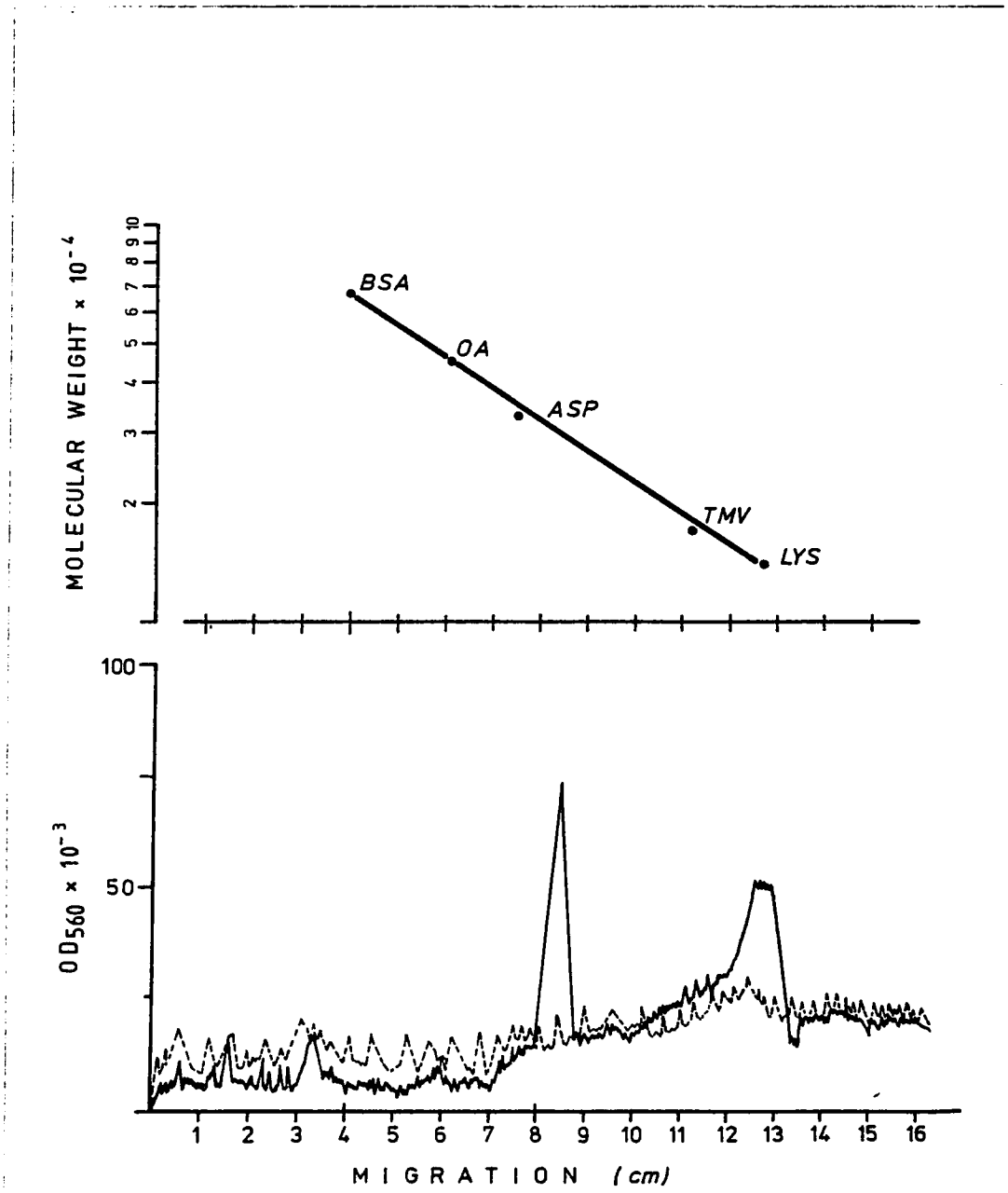

FIGURE 14. Analytical SDS-PAGE of schemes 1 and 2 MIF. The degree of purity of schemes 1 (——) and 2 (---) MIF were analyzed by SDS-PAGE. The molecular weight standards were bovine serum albumin (BSA) MW 67,000, ovalbumin (OA) MW 45,000, asparaginase (ASP) MW 33,000, tobacco mosaic virus (TMV) MW 17,000, and lysozyme (LYS) MW 14,000.

DISCUSSION

The substantial purification of mouse interferon achieved by CPG-adsorption chromatography can be attributed to : (i), the ability of MIF to bind to CPG, and (ii), unlike the majority of other CPG-bound proteins, to elute under acidic conditions (43, and results presented here).

A variety of low pH buffers were tested for either their ability to elute the maximum amount of protein, and the minimum of interferon, or the converse (i.e. to elute the maximum interferon, and minimum protein). Obviously, the need for the first buffer precedes the second. Although no significant differences were discerned between the combinations 10 mM gly-HCl, pH 2.5, followed by 100 mM KCl-HCl, pH 2.0, and 100 mM gly-HCl, pH 3.5, followed by 100 mM gly-HCl, pH 2.0, the latter elution scheme was followed mainly for three reasons : first, regardless of the purity of the starting material (i.e. crude or 1°-MIF), the bulk of the MIF activity eluted within a narrow pH range (2.3 - 3.2) and therefore was easily identifiable; second, the author's preference for continuity in buffers; and lastly, avoidance of the chaotropic effects of KCl on more highly purified preparations of MIF.

Comparison of the results obtained with crude or 1°-MIF as the starting material, showed that, under the same conditions, a higher specific activity was obtained with the latter than with the former. One must assume therefore, that $(NH_4)_2SO_4$ precipitation eliminated some of the proteins which otherwise co-elute with MIF from CPG. The first step also reduced the amount of CPG-bound, alkali-elutable proteins (data not shown).

Although the majority (94 %) of the CPG-bound proteins were removed under alkaline conditions, as was observed from the protein elution profiles, no further significant purification of MIF was achieved. This suggests an independent assortment of proteins bound to CPG, one acid-elutable (43), the other alkali (11,86). Furthermore, in general terms, at least with interferon preparations, one class of proteins would not elute with another.

Occasionally (see Chapter 2), some interferon (less than or equal to 1 %), could be detected in the alkali wash. Pending further scrutiny, this sporadically occurring phenomenon can be attributed to "heterogeneity" of the interferon preparations. Subsequent purifications did not include alkali pre-washes for reasons described above, and the concern for the stability of more purified MIF to high pH.

Regardless of the elution procedure employed, crude MIF could be purified 40-100-fold, by CPG-adsorption chromatography, with recoveries varying from 24 to 100 %. In addition, when 1°-MIF was applied (specific activity 2.5×10^5 units/mg), a specific activity of 5.0×10^7 units/mg was obtained.

At least two classes of proteins elute from CPG, and their mechanisms of binding are, most likely, different. The surface of the beads contains essentially only two binding sites, silanol and B_2O_3 groups (136). Both require, for protein binding, the presence of amino groups. If silanol, via its hydroxyl groups, were the responsible ligand at physiological conditions, one could predict a binding between its negative charges (96) and the protein's positively-charged amino acids. To some extent, this hypothesis was borne out by the fact that an increase in negative charge of the beads (by increasing the pH), did not lead to a release of interferon.

The role of lysine and arginine residues in MIF-CPG interactions were first studied by reversibly blocking either the protein's ε-amino groups (of lysine) or guanidinium groups (of arginine) with citraconic anhydride. The citraconylated MIF did not bind to CPG. Although this observation points to the necessity of lysine and arginine residues in the binding of MIF to CPG, one cannot discount the possibility that the citraconylated MIF, as other citraconylated proteins, assumed a conformation which masked the CPG-binding sites. Both events are, of course, not mutually exclusive.

To further substantiate the role of lysine and arginine, the elution profiles of poly-l-lysine and poly-l-arginine from CPG, were also examined. Similar to MIF, bound poly-l-lysine eluted under acidic, but not alkali, conditions. Unlike MIF, however, half of the applied material did not bind. This event is difficult to explain considering that the polymer was characterized by the manufacturer as homogeneous (with molecular weights varying from 15,000 to 30,000, as determined by viscosity), that all of the applied material was recovered in either the drop-through or acid-washes, and that all of the material (i.e. applied, drop-through or acid-wash) had the same spectra, peaking at 208 nm (data not shown). Two possibilities, however, are lack of sufficient column capacity and physical restrictions. As reducing the amount of sample loaded did not significantly alter the results, the first possibility can be discounted. Although the polymer's molecular weight was within the size limitation of the beads (4.0×10^4 - 4.0×10^6),

the exclusion limits apply only for globular proteins and spherical viruses. Under physiological conditions, poly-l-lysine assumes an alpha-helical conformation and thus can be of greater size than the viscosity determination would reveal. Consequently, because it is the area within the pores which has the highest binding capacity (V.G. Edy, personal communication), some of the polymers may have remained unbound. Furthermore, some of the α-amino groups may hydrogen bond to the α-keto groups, and therefore, a portion of the poly-l-lysine molecules may obtain different binding characteristics.

Except for the two peaks of homopolymer detected during the alkali-wash, poly-l-arginine exhibited the same elution profile as poly-l-lysine. Since some of the material eluted under alkali conditions one might conclude that the binding and elution characteristics for this homopolymer is more complex. Furthermore, the bi-modal elution profile observed during the alkali-wash can be best explained by considering the differences in pH of the eluant. Despite the step-wise elution procedure employed, the pH of the early washes were lower than the latter. The same explanation provided for the drop-through material of poly-l-lysine, most likely, also applies to poly-l-arginine.

In as much as the pK of the α-amino group of lysine is 8.95, it is unlikely that either MIF or poly-l-lysine binds to CPG from this location. However, it is tempting to speculate that binding could occur from the terminal α-carboxyl group (pK = 2.18), and whether conformations of citraconylated proteins shift so as to mask this group.

More than the presence of lysine and arginine must be required for binding, since all proteins contain these ubiquitious amino acids. Therefore regions of high basic amino acid content may be the explanation. This is supported by the fact that MIF is an extremely basic protein, with an isoelectric point (pI) of at least 9.5 (62). Furthermore, because of this high pI and that MIF elutes from ionic exchange resins at a pH greater than 6.0 (61,67, and the results obtained here with CM-BGA), the protein-bead interactions cannot simply be an ionic exchange phenomenon.

Two amino acids, glycine and alanine, have been reported to prevent the adsorption of albumin to CPG (85). The role of the former in MIF-CPG interactions (via hydrogen bonding) could be discounted for the following reasons: first, 400 mM glycine did not remove MIF from the beads, unless the pH was less than 3.5; and second, the presence of high salt, including

the chaotropic agent KCl, did not elute MIF, except when the pH was less than 3.5. The possible role of alanine *via* hydrophobic interactions, is discussed below.

The elution conditions employed, and the degree of purification obtained with ZrOH, suggests that the B_2O_3 groups, *via* Lewis acid-base interactions, do not play a major role in MIF-CPG binding. In addition, the absence of silicon implies that, for MIF binding to occur, the beads require hydroxyl groups only.

Unfortunately, ZrOH, as a means of purifying MIF, is not practical. Unlike the ridged structure of CPG, ZrOH assumes a powder-like form, and consequently, when packed in a column, delivers an extremely slow flow rate. When employed in a batch process, the capacity of the polymer is rather limited, and as a consequence a substantial amount of MIF does not bind.

In view of the results obtained with citraconylated MIF (where only sites containing the ε-amino groups of lysine and/or the guanidinium groups of arginine are altered), and ZrOH, which provides no Lewis acid-sites, the binding of MIF *via* its terminal primary amino groups (or Lewis bases) of arginine, asparagine, or glutamine can be discounted.

MIF also elutes from CPG in the presence of the hydrophobic buffer PBS containing 10 M ethylene glycol and 1 M NaCl (EG + NaCl). The presence of 1 M salt alone does not desorb MIF (data not shown), therefore, both, or EG alone were required. It is not clear however, whether the buffer affected the protein and/or protein-bead interactions, or as polyethylene glycol indiscriminately removes all bound proteins (96), the beads. As, when first washed with EG + NaCl, fewer, but not all proteins (2.2 % of the recovered material) eluted with the alkali buffer, than if EG + NaCl were not used (6.9 % of the recovered material), a combination of both events seems the most likely. This data, however, also suggests that since some alkali-elutable proteins were removed with EG + NaCl, the elution is not specific for acid-elutable, (including MIF), proteins. The minute amount (0.05 % of the starting material) of MIF which eluted only under acidic conditions can also be best attributed to interferon's heterogeneity.

The binding interactions between MIF and CPG are therefore presumed to be between some of the protein's ε-amino groups of lysine and/or guanidinium groups of arginine and the hydroxyl groups of the beads. A hydrophobic interaction, possibly *via* such residues as alanine, also serves, perhaps less specific, a role. MIF's elution could result from either a shift in protein

conformation (60), or charge on the beads. Since the pK of the silanol groups is between 6 and 8 (59), the latter seems likely.

Similar to many proteins, MIF covalently binds to Agarose-p-aminophenyl mercuric acetate (Hg-agarose). However, as seen from the protein elution profile, not all protein neighbors of MIF possess cysteine (as most is converted to cystine), and/or contain it within a region accessible to binding. Although not useful as a method for MIF purification, organomercurial affinity chromatography did serve to indicate that MIF can be classified as containing accessible cysteine residues. Similar results were obtained when 50 mM NaAc, pH 5.0, containing 1 % 2-mercaptoethanol was employed as an eluant (data not shown), thus verifying the formation of mercaptide bonds between MIF and Hg-agarose.

MIF has been previously been shown to be purified by hydrophobic chromatography with AFFI-Gel 202 (33). In our experiments however, where the purity of the starting material was higher, the final specific activity (3.2×10^6 units/mg) of the interferon product was less than that reported earlier. Nevertheless, the technique did offer some addition purification, and proved quite useful when employed in concert with other techniques (see below).

Many groups have previously reported the use of gel filtration as a means of purifying MIF (61,62,67). This is the first attempt, however, to employ Ultrogel AcA 54. The gel, a mixture of polyacrylamide and agarose, offered both good recoveries (12 to 123 %) and purification (30-fold, with a final specific activity of 3.0×10^7 units/mg).

Although we used the buffer system previously described by Knight (67), neither glycerol, which contains impurities that have a tendency to cross-link proteins (8), nor salt, appeared to be necessary, despite the low protein content of the applied material. The deletion of the salt allowed direct loading of the eluted material onto CM-BGA, without the need of a dialysis step (67).

It is noteworthy that the molecular weight of 2°-MIF (Figure 11) was, for material similarly prepared and endowed with the same purity, lower than previously reported (67). This may have been due to either differences in the inducer, or the exclusion limits of the gel. With the mouse interferon studied in this report, Ultrogel AcA 54 exclusion limits (5,000 - 70,000 daltons) offered the best resolution.

The use of ionic exchange chromatography has also been employed by many groups (38,61,67,69,81). The procedure outlined in this study utilized the new exchanger Carboxymethyl Bio-Gel Agarose (CM-BGA), which yielded high recoveries (64 %), and if loaded with material first purified by gel filtration (see above), a product with a specific activity of 6.2×10^7 units/mg, or, starting with 1°-MIF, a 238-fold purification. Although the buffer system of Knight (67) was employed, with this resin, for reasons mentioned above, the presence of glycerol could be eliminted, despite the low protein concentration of the starting material.

The major obstacles in purifying interferons are, at low protein concentrations, its tendency to become inactive (67,69), and/or nonspecifically bind to the apparatus employed (119). These difficulties might be overcome in two ways : begin with a sufficiently high amount so that the product's protein content continues to sustain activity (18,62,67,69); or, design a procedure which requires the minimum amount of time,steps, and/or manipulations (e.g. dialysis and concentration). The latter, has shown to be successful by De Maeyer-Guignard et al. (37), and when compared to another procedure giving the same specific activity (62), to be less tedious and more efficient.

Our attempts have also been directed at designing a more versatile method of interferon purification. The overall strategy was to judiciously select efficient purification techniques where the effluent and affluent buffers could be coordinated, and thereby reduce both the number of manipulations and time, required. The first (scheme 1), involved two affinity/adsorption techniques, hydrophobic chromatography and adsorption chromatography with AFFI-Gel 202 and CPG, respectively. The only manipulation required was a previous dialysis following $(NH_4)_2SO_4$ precipitation. The final product had a specific activity ranging from $1.0 - 8.0 \times 10^8$ units/mg, and, when beginning with 1°-MIF material, represented a 16 - 116-fold purification. The recovery (59 %) was comparable to that of other groups reporting the same specific activity, but in our experiments significantly less starting material was required (61,67). When started with the dialyzed material, the actual purification steps usually took no more than one day.

As an extension of the above technique, purification scheme 2 was offered. In this scheme, MIF was first purified by physicochemical means (i.e. gel filtration with Ultrogel AcA 54 followed by ionic exchange chro-

matography with CM-BGA). Except for having to dialyze the 1°-MIF, the only manipulation required was the dilution and pH readjustment of 3°-MIF. Despite the low recovery (7.4 %), the final product had a specific activity varying from 2.5 - 3.7 x 10^8 units/mg and, when beginning with 1°-MIF, represented a 19 - 44-fold purification.

Judging from the final products (both schemes 1 and 2) low purification factor, the high degree of purity obtained was most likely a result of both the methods employed and the purity of the starting material. Furthermore, considering the low recoveries obtained from scheme 2 products, and the inability to detect any protein on SDS-PAGE, it is difficult to determine whether, ultimately, scheme 2 offers any additional purification compared to scheme 1.

The discrepancy between the molecular weights of MIF as determined by gel filtration (11,000 - 12,000 daltons, Figure 11) and SDS-PAGE (39,000 - 44,000 daltons and, with scheme 1 material also, 19,000 - 23,000 daltons; Figure 13) may reflect either differences in technique or the bifunctionality of SDS. The results, with SDS-PAGE, obtained herein, were very similar to previous reports (37,62,67,118). Furthermore, in the past, both of these techniques rendered the same molecular weight determinations for interferons (23,138). Thus the differences in molecular weight found in this report would appear to be the result of the gel filtration procedure. Indeed, regardless of the technique (including gel filtration), this is the lowest molecular weight determination reported for mouse interferon. However, in no investigation were the conditions for interferon production or gel filtration identical. Although we employed the buffer described by Knight (67), the method of MIF induction, and the gel matrix used were different. The presence of glycerol, in the Knight procedure (67), which contains impurities that have a tendency to cross-link and modify proteins (8), may also have been the critical factor. Alternatively, MIF, as it is a glycoprotein, may drag on this resin and not on others.

Although the possibility is remote, as SDS is a bifunctional molecule (i.e. contains a hydrophobic and polar region, see Chapter 2), the presence of SDS, in the PAGE, may have served as an inter-molecular bride between MIF, other MIF molecules, or some of the contaminants still present.

As shown in Figure 13, the appearance of the low molecular weight component of MIF (i.e. 19,000 - 23,000 daltons) will vary from preparation to

preparation (E. De Maeyer, personal communication).

The molecular weight ranges from MIF (determined on preparative gels) did not coincide with the protein bands detected, for scheme 1 products, on analytical gels. This suggests that, despite the high degree of purification, MIF was not the predominant protein. Interestingly, the results obtained from the analytical gel did reveal a low molecular weight ($<14,000$ daltons) protein. Although Kawakita et al. (62) have described such a component as being inert, in light of the low recoveries obtained from the SDS-PAGE gels in this report, and the appearance of a low molecular weight species of mouse interferon as seen by gel filtration (see Figure 11), it is tempting to speculate whether this band represents inactive interferon.

SUMMARY

1. Mouse interferon (MIF) can be purified, under a variety of elution techniques, by Controlled Pore Glass (CPG)-adsorption chromatography. When crude (specific activity 2.5 - 4.1 x 10^4 units/mg) material was applied, a 40 - 100-fold purification could be achieved, with recoveries ranging from 24 to 100 %. Furthermore, if more purified (specific activity 2.5 x 10^5 units/mg) preparations were applied to the CPG columns, a specific activity of 5.0 x 10^7 units/mg could be obtained. Regardless of the starting material, MIF eluted from CPG within a narrow pH range (2.3 - 3.2).

2. MIF binds to CPG via some of the protein's ε-amino groups (of lysine) and/or guanidinium groups (of arginine) and the beads silanol (hydroxyl) groups. A requirement for Lewis acid-base interactions between the beads B_2O_3 groups and the terminal primary amino groups of arginine was discounted in view of the results obtained with citraconylated interferon (which did not bind to CPG) and the alternative binding system, ZrOH (which is devoid of B_2O_3). Since a substantial amount of interferon could be eluted from CPG with ethylene glycol, one may assume that some hydrophobicity is involved in the binding of MIF to CPG.

3. As determined by organomercurial affinity chromatography, MIF belongs to those proteins which not only contain cysteine, but also whose thiol groups are accessible to organomercurial binding sites.

4. Employing a new gel filtration technique, MIF was purified with Ultrogel AcA 54 30-fold (specific activity 3.0 x 10^7 units/mg), and with recoveries ranging from 12 to 123 %. Unlike Bio-Gel P-150, which had been previously described for interferon purification (67), Ultrogel AcA 54 did not require the presence of glycerol or salt. Hence, with the elimination of the requirement for salt, the Ultrogel AcA 54 elute could be directly loaded onto other columns without the need of dialysis. MIF eluted from Ultrogel AcA 54 within a lower molecular weight range (11,000 - 14,000 daltons) than has previously been reported with other gel filtration techniques.

5. When further processed by Ultrogel AcA 54 chromatography followed by ionic exchange chromatography on Carboxymethyl (CM)-Bio Gel Agarose,

MIF eluted from Ultrogel AcA 54 could be purified (starting with 1°-MIF) 238-fold, to a specific activity of 6.2×10^7 units/mg, and with 64 % recovery.

6. MIF could also be purified to a final specific activity of 1.0 - 8.0 x 10^3 units/mg when first precipitated with $(NH_4)_2SO_4$, and further processed by hydrophobic chromatography and adsorption chromatography, on AFFI-Gel 202, and CPG, respectively. Although the recovery attained (59 %) was comparable to that reported for other procedures yielding the SAME specific activity (61,67), the approach followed here required less starting material. The final product, when analyzed by SDS-Polyacrylamide Gel Electrophoresis (PAGE) had two peaks of activity, one ranging between 39,000 - 44,000 daltons, and the other 19,000 - 23,000 daltons.

7. MIF could also be purified to a final specific activity of 2.5 - 3.7 x 10^8 units/mg when first precipitated with $(NH_4)_2SO_4$ and then further processed by gel filtration with Ultrogel AcA 54, ionic exchange chromatography with CM-Bio Gel Agarose, hydrophobic chromatography with AFFI-Gel 202, and CPG-adsorption chromatography. The final product, when analyzed by SDS-PAGE had only one peak of activity which ranged between 39,000 to 44,000 daltons.

CONCLUDING REMARKS

The Ph.D. thesis research could be divided into three categories : the purification, immunochemical, and chemical characterization of mouse interferon. The chemical characterization was essentially based on a study of the interactions between sodium dodecyl sulfate (SDS) and mouse interferon.

The immunochemical and functional similarities between mouse and human (fibroblast and leukocyte) interferons were examined. By studying the degree of neutralization of interferon by homologous and heterologous anti-interferon antibodies, when assayed on homologous and heterologous cells, it was determined that stronger binding occurred for homologous interferon-cell receptor pairs than for heterologous pairs. From this data, a multiple-fit model was proposed whereby, with respect to cell receptors, all interferons share partially similar active sites and that the extent of antiviral activity conveyed is a function of the closeness of fit between cell receptor and interferon.

The interactions between SDS and mouse interferon were investigated from three perspectives : (i), the physicochemical characteristics of the amphiphile required to stabilize interferon; (ii), the binding interactions between SDS and interferon as studied by ionic exchange, Controlled Pore Glass (CPG)-adsorption, and hydrophobic chromatography; (iii), and differences between SDS-treated and untreated mouse interferons' susceptibilities to antibodies and heat. The results suggested that, in order to stabilize mouse interferon, a twelve-membered (or higher) carbon alkyl chain, with a terminal polar sulfate group, was required. Furthermore, while at least some of the hydrophobic and polar regions of the interferon-bound amphiphiles appear to be located on the surface of the protein, their association with interferon did not alter either interferon's antigenicity or thermolability.

The purification of mouse interferon was achieved by employing two concert chromatographic techniques. With both procedures the conditions for adsorbing and desorbing interferon were coordinated so as to reduce the amount of time and number of manipulations (e.g. dialysis, concentration) required for purification, and thus diminish the possibility of inactivation. The starting material was ammonium sulfate-precipitated interferon.

The first purification scheme employed hydrophobic chromatography with AFFI-Gel 202, and CPG-adsorption chromatography. The second procedure involved gel filtration chromatography with Ultrogel AcA 54, ionic exchange chromatography with CM-Bio Gel Agarose, followed by the procedures described in the first scheme. With the former scheme, mouse interferon was purified 16 - 114-fold to a specific activity of $1.0 - 8.0 \times 10^8$ units/mg. With the latter, mouse interferon was purified 19 - 44-fold to a specific activity of $2.5 - 3.7 \times 10^8$ units/mg.

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